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LA SPÉCIFICITÉ DANS LA SYMBIOSE *RHIZOBIUM*-LÉGUMINEUSES

FAITS EXPÉRIMENTAUX, CONCERNANT LA GENÈSE DE LA NODULATION

par

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INTRODUCTION.

Le problème de la spécificité, dans la symbiose *Rhizobium*-Légumineuses, est resté jusqu'à ce jour presque entièrement posé.

Les différentes souches bactériennes, appartenant au genre *Rhizobium*, ne peuvent le plus souvent se différencier que par l'électivité et l'efficacité de leurs propriétés symbiotiques. En outre, entre les genres *Rhizobium* et *Agrobacterium*, de la même famille des *Rhizobiaceae*, la seule distinction possible réside dans leurs propriétés, soit saprophytiques ou parasitaires pour *A. radiobacter* et *A. tumefaciens*, soit symbiotiques pour *Rhizobium*.

Cette conclusion résulte notamment des observations morphologiques, biochimiques et antigéniques que nous avons faites (5). Nous basant sur ces caractères de différenciation, il nous a été impossible d'établir une classification des *Rhizobium*, correspondant avec la spécificité de l'hôte.

Citons, parmi les observations qui soutiennent notre opinion : a) le critère qui consiste à différencier des souches de *Rhizobium* spécifiques de *Soja hispida* par exemple, par la lenteur et la pauvreté du développement sur le milieu de WRIGHT (27), ne correspond pas à la réalité. Nous avons isolé des souches de *Rhizobium* spécifiques de *Soja hispida* dont le développement est rapide et abondant.

b) les deux groupes obtenus en lait tournesolé, selon la réaction

¹⁾ Associé du Fonds National Belge de la Recherche Scientifique.

observée, n'ont pas de rapport avec la spécificité de l'hôte. Deux souches spécifiques de la même espèce Légumineuse peuvent présenter, l'une une réaction acide, l'autre une réaction alcaline. Or, ce caractère est précisément à la base de la classification de BERGEY (8). c) deux souches de *Rhizobium* spécifiques de la même espèce peuvent être nettement différenciables par leurs caractères antigéniques, alors que des souches différant dans leurs propriétés symbiotiques peuvent être indiscernables. Il s'agit là d'un fait bien établi (21). d) les inoculations nombreuses que nous avons faites infirment la valeur du test "inoculations croisées", en vue d'une classification des diverses souches de *Rhizobium*. WILSON d'ailleurs (26) était arrivé dès 1939 à des conclusions analogues.

Si nous examinons maintenant les rapports du *Rhizobium* avec les espèces *A. radiobacter* et *A. tumefaciens*, également de la famille des *Rhizobiaceae*, nous ajouterons qu'il existe des races de *Rhizobium* qui ont perdu non seulement leur capacité de fixer l'azote ("effectivité") mais encore leur pouvoir de former des nodules ("infectivité"). Il existe de la même façon des souches d'*A. tumefaciens*, ayant perdu, naturellement ou expérimentalement, leur pouvoir pathogène pour la plante.

Du fait que certaines races de *A. radiobacter* et de *A. tumefaciens* ne peuvent être séparées, ni morphologiquement, ni biochimiquement, ni antigéniquement, de certains *Rhizobium*, nous pouvons affirmer que les trois organismes, *Rhizobium*, *A. radiobacter* et *A. tumefaciens* sont, dans certains cas, indiscernables.

Pour être complet, ajoutons qu'il n'existe pas non plus de spécificité bactériophagique absolue.

Nous croyons donc qu'il est vain de vouloir classer les bactéries du groupe *Rhizobium* en différentes espèces, puisque le seul caractère que l'on puisse leur donner avec certitude réside dans leur origine. Cette situation explique facilement, par la diversité des conditions de travail, toutes les observations contradictoires qui ont été effectuées.

A notre avis, il n'existe pas 2, 6, 9, 12 ou 32 espèces de *Rhizobium* (selon les auteurs), mais bien un vaste groupe de bactéries, possédant des caractères communs, et capables, dans certaines conditions, de vivre en symbiose sur les racines de Légumineuses, en fixant l'azote atmosphérique. Ce groupe est constitué de formes en perpétuelle évolution, évolution liée à l'influence de facteurs internes ou externes (sol, plantes, conditions climatiques).

Encore convient-il de s'expliquer à propos de cette perpétuelle évolution des "formes" de *Rhizobium*. Dans les conditions naturelles de très nombreuses espèces Légumineuses sont colonisées, alors qu'au laboratoire, par la technique des inoculations croisées, en conditions aseptiques, nous ne pouvons obtenir des nodules sur une espèce végétale inoculée au moyen d'une souche isolée d'une autre espèce Légumineuse, que dans certains cas seulement.

Dès les premières observations que nous avons faites, relatives à ce problème de la spécificité, il nous est apparu que diverses Légumineuses pouvaient, dans les conditions naturelles, être colonisées par des types variés de *Rhizobium*, à la suite d'un processus, disons d'adaptation, ce terme étant utilisé dans son sens le plus large.

Si nous ne sommes pas à même de détailler les mécanismes de cette adaptation, nous pouvons rapporter tout au moins les faits suivants, qui ont servi de point de départ à notre nouvelle étude: a) Soit un sol naturel, non stérilisé, qui contient des formes quelconques de *Rhizobium*, capables de former des nodules sur une série de Légumineuses. Sur le sol ainsi défini, semons une espèce Légumineuse bien déterminée, qui n'y a jamais été cultivée et qui n'y forme pas de nodosités. Si nous pratiquons sur ce sol des semis successifs, c'est à dire si nous cultivons successivement plusieurs fois la Légumineuse en cause sur le même sol, nous obtiendrons, après 3 ou 4 semis, des nodules en nombre de plus en plus élevé, pour finalement obtenir une fixation intense d'azote. Nous avons vérifié le fait à maintes reprises et notamment sur *Soja hispida*, (3), sur *Arachis hypogaea*, (4), sur *Medicago sativa*, *Phaseolus spec.* et *Pisum sativum*. Plus récemment, en 1956, SARIC (22) a étudié "l'adaptation naturelle des *Rhizobium* d'un sol aux Légumineuses *Lespedeza*, *Soja*, *Glycine*, *Arachis* et *Phaseolus lunatus*". Les observations de cet auteur confirment celles que nous avons faites précédemment. RIEDE, cité par JOURNÉE et TILKIN (11), estime que le fait de cultiver le *Soja* trois fois sur lui-même, améliore largement l'état de la culture et assure les plus hauts rendements. On peut ajouter qu'en certaines régions de l'Amérique du Sud, les autochtones savent qu'un premier semis de *Soja*, sur sol vierge, ne donne jamais de récolte appréciable. Ils ont observé que, par contre, s'ils continuaient à semer la même espèce sur le même sol, ils finiraient par obtenir une récolte excellente. (Ce fait constitue par ailleurs une parfaite démonstration de l'utilité de l'inoculation préalable des graines).

Ce cas des semis successifs, que nous venons de rapporter, est

explicable par la sélection, dans le sol, de mutants bactériens spécifiques. Cette explication, nous allons le voir, est insuffisante pour éclairer d'autres faits.

b) *Trifolium ambiguum* M. Bieb est une espèce fort intéressante au point de vue agronomique. Espèce rustique, résistante aux froids et à la sécheresse, elle se multiplie facilement par stolons et peut être pâturée tout en assurant une excellente repousse. Ce *Trifolium ambiguum*, originaire du Caucase, a été introduit aux Etats-Unis, où l'on s'est aperçu qu'il ne portait que de très rares nodules dans les sols étudiés. En offrant une grande résistance à l'invasion par le *Rhizobium*, cette espèce fait exception en quelque sorte dans le genre *Trifolium*.

En collaboration avec HELY et MANIL, (9) nous avons imaginé de greffer sur *T. ambiguum* du *T. repens* et du *T. pratense*. Par cette méthode, nous avons obtenu des nodules sur *T. ambiguum* alors que les plants témoins, non greffés, en étaient toujours dépourvus dans les sols belges considérés.

c) Nous avons également entrepris des essais de greffes intergénériques (6,7). Le terme de greffe est employé ici abusivement, pour une opération qui consiste à faire vivre, sur une tige de *Soja hispida* par exemple, des greffons de *Trifolium*, *Medicago*, *Pisum*, *Phaseolus*, *Vicia* etc. Il ne s'agit pas de greffes au sens strict, puisque, jamais, nous n'avons obtenu la soudure parfaite des tissus. Mais l'expérience a montré qu'il était possible de cultiver sur une tige de *Soja hispida* des greffons pris sur d'autres genres de Légumineuses. Le greffon, en bonnes conditions de température et d'humidité, persiste longtemps sur le sujet et forme même parfois de nouvelles racines qui s'insinuent dans la tige de celui-ci. Des échanges sont rendus possibles, jusqu'à un certain point tout au moins. Ainsi, si on greffe sur *Soja* cultivé dans des vases dépourvus de souches spécifiques, un greffon de *Medicago*, de *Trifolium* ou d'autres espèces, dont les souches spécifiques sont présentes dans les vases de culture, on obtient des nodules sur *Soja*, alors que les *Soja* témoins non greffés, cultivés dans les mêmes vases, en sont toujours dépourvus. De plus, les souches isolées de *Soja* greffés, sont capables d'infecter cette fois des *Soja* non greffés.

d) Tous les essais effectués pour tenter d'obtenir des nodules sur des cultures de racines de Légumineuses, dépourvues de toute partie aérienne et inoculées au moyen de la souche spécifique, ont jusqu'à présent échoué, à notre connaissance tout au moins.

Ceci montre bien, comme les essais de greffes rapportés ci-dessus, que les constituants biochimiques de la plante-hôte conditionnent, de façon certaine, la formation de nodules à partir d'une souche déterminée de *Rhizobium*. Une race bactérienne est spécifique des *Trifolium* par exemple, parce qu'il y a compatibilité, correspondance, entre cette souche et la constitution biochimique de la plante-hôte.

D'un autre côté, les travaux de NUTMAN (15-18) ont démontré expérimentalement que les méristèmes apicaux des racines produisaient des substances inhibant, à des degrés divers, la formation de nodules par des souches spécifiques, ainsi que la formation de nouvelles racines latérales. Ces substances inhibitrices sont excrétées par les racines, puisque NUTMAN a observé que l'influence de la plante sur le nombre d'infections s'exerçant au-delà des racines (les plantes croissant en association ne réagissant pas de la même manière que les plantes croissant seules). THORNTON (23) ainsi que LUDWIG et ALLISON (13) envisagent, en dehors de ces substances inhibitrices, la production par la plante également, de substances stimulantes. (Sur *Medicago sativa* par exemple, le premier nodule ne peut jamais se former avant l'apparition de la première vraie feuille).

Ces actions de stimulation et d'inhibition pourraient d'ailleurs être dues à un même composé, dont les effets varieraient avec la concentration. Cette question vient tout naturellement à l'esprit puisque NUTMAN (16) a montré que lorsque des jeunes plants de *Medicago* sont cultivés en association dans un milieu limité, il y avait stimulation de l'infection chez les toutes jeunes plantules et réduction du nombre de nodules sur les plus vieilles plantes.

Ainsi donc, ces composés originaires de la plante exerceraient une action décisive sur la formation du nodule ainsi que sur son comportement ultérieur. Selon THORNTON (23), il y a trois processus distincts dans la vie du nodule :

- 1) croissance du méristème apical,
- 2) invasion successive de ses couches internes par la bactérie,
- 3) désintégration finale qui débute à la base du tissu bactérien.

Il est essentiel que les vitesses relatives de ces trois processus soient bien ajustées, si le nodule doit fonctionner effectivement un certain temps.

Si le méristème apical croît trop lentement pour échapper à l'infection, les bactéries envahissantes arrêtent la division cellulaire;

le nodule restera petit et son centre sera bientôt détruit par le progrès de la désintégration. Par contre, si le méristème apical se forme à une vitesse suffisante pour échapper à l'infection, le nodule peut fonctionner un temps plus ou moins long, variable avec la vitesse de désintégration des tissus centraux.

On voit de suite le rôle essentiel que joueraient ces substances stimulantes et inhibitrices dont l'action conjuguée équilibre la nodulation, en réglant les vitesses de formation et de désintégration du nodule. Conséquence importante de cette action de régulation: elle influe sur l' "effectivité" du nodule.

Ces actions stimulantes et inhibitrices, il faut le noter, ont été observées par NUTMAN et THORNTON, au cours d'essais où une Légumineuse déterminée était toujours inoculée au moyen d'une souche de *Rhizobium* spécifique.

Dans le cadre de nos travaux sur la spécificité, nous nous sommes demandé s'il n'était pas possible de rendre "infective" une souche de *Rhizobium* non spécifique, en bloquant, par l'un ou l'autre procédé, les excrétions radiculaires.

Puisque ce sont ces excrétions qui règlent la nodulation par une souche spécifique, nous avons pensé que l'infectivité d'une souche non spécifique pouvait être conditionnée par le blocage de ces composés. En bref, nous nous sommes demandé s'il était possible d'obtenir des nodosités sur les racines de Légumineuses, inoculées au moyen d'une souche non spécifique (incapable en conditions normales de provoquer la nodulation), en adsorbant les excrétions radiculaires.

MATÉRIEL ET MÉTHODES.

Comme adsorbant des excrétions radiculaires, nous avons utilisé le charbon de bois.

Ce composé avait déjà été utilisé par VANTSIS et BOND (23), par NUTMAN (19) et par TURNER (24).

Ces auteurs ont cultivé, soit en vases de végétation, soit en tubes à essai des plantules de *Pisum* et de *Trifolium*, en présence de charbon de bois. Dans tous les cas, les espèces Légumineuses étaient inoculées au moyen d'une souche spécifique.

Cette technique n'avait donc jamais été utilisée pour des essais concernant la spécificité.

Selon VANTSIS et BOND (23), l'addition de petites quantités (0.5

à 2 pour mille) de charbon de bois au milieu radiculaire de plantes de *Pisum* inoculées, dans une culture sur sable dépourvu d'azote, provoque une augmentation marquée du poids sec des plantes et de la fixation d'azote. Selon ces auteurs, le nombre de nodules est réduit, mais leurs dimensions sont très nettement augmentées, ce qui a pour résultat définitif un accroissement de fixation d'azote.

Cette action favorable du charbon de bois est attribuée soit à une adsorption d'excrétions radiculaires défavorables, soit à une action sur le pH du milieu.

L'augmentation de la fixation d'azote est vérifiée par analyse Kjeldahl. Est-ce vraiment la fixation d'azote qui est accrue? VANTSIS et BOND ne signalent pas que le charbon de bois peut contenir une certaine quantité de nitrate. Nous avons dosé l'azote nitrique, dans un charbon de bois non purifié, tel que celui qui fut utilisé dans nos essais; il peut contenir 0.22 mg /gramme de NO_3 . NUTMAN (19) a travaillé sur des plantes de *Trifolium*, cultivées en tubes à essai. Cet auteur lui aussi a observé une fixation d'azote plus élevée mais signale, contrairement aux auteurs précédents, que le nombre de nodules est sensiblement augmenté. NUTMAN ne signale pas, lui non plus, que l'enrichissement des plantes en azote pourrait être dû au nitrate contenu dans le charbon de bois.

Pour TURNER (24), le nombre de nodules est très nettement augmenté sur des plantules de *Trifolium* cultivées en tubes à essai.

Il est remarquable que, dans l'essai de TURNER, le charbon de bois, déjà utilisé, a fourni des extraits, à partir de solvants organiques, qui pouvaient, soit stimuler, soit inhiber la production de nodules. L'auteur attribue cette action favorable ou défavorable à la concentration des sécrétions radiculaires dans les éluats, ce qui pourrait confirmer l'hypothèse émise plus haut: un même composé produit par les plantes pourrait avoir des effets stimulants ou inhibiteurs, suivant la concentration.

Tous ces auteurs d'ailleurs sont d'accord pour attribuer, dans leurs essais, l'action stimulante du charbon de bois à ses propriétés d'adsorption des composés inhibiteurs secrétés par la plante. Si ces composés inhibiteurs agissent ainsi vis à vis d'une souche de *Rhizobium* spécifique, leur action peut être plus marquée encore vis à vis d'une souche non spécifique, incapable de provoquer la nodulation.

C'est précisément cette idée qui a servi de point de départ à notre travail.

Essais proprement dits:

Nous avons cultivé aseptiquement en tubes à essai (180×16 mm) des plantules de trois espèces Légumineuses, sur le milieu synthétique suivant, de NICOL et THORNTON (14):

K_2HPO_4	0.5 g	$Ca_3(PO_4)_2$	2 g
$MgSO_4 \cdot 7 H_2O$	0.2 g	$FeCl_3$	0.01 g
NaCl	0.1 g	Agar	15 g
$FePO_4$	1 g	Eau de conduite	1 litre

Les semences sont désinfectées selon notre procédé habituel, par trempages successifs dans l'alcool 96° (1 minute), dans $HgCl_2$ 2,5 pour mille (5 minutes) et dans 10 bains de rinçage à l'eau stérile. Les graines ainsi traitées sont mises à germer en boîtes de Petri sur le milieu riche de WRIGHT (27), qui permet d'éliminer les plantules non stériles.

Après 48 heures de germination, les plantules sont transférées aseptiquement en tubes contenant le milieu nutritif incliné. Du charbon de bois en poudre, "Norit", stérilisé au four, était réparti en tubes contenant 1 plantule, sur toute la surface inclinée, à raison de 1 cc par tube contenant 10 cc de milieu nutritif. Les tubes étaient inoculés au moyen de 1 cc d'une suspension aqueuse de bactéries.

Dans un premier essai: les tubes suivants ont été préparés:

- 12 tubes contenant 1 plantule de *T. pratense*, avec charbon de bois, non inoculés,
- 12 tubes contenant 1 plantule de *T. pratense*, avec charbon de bois, inoculés au moyen de la souche S. 50, spécifique des *Trifolium*,
- 12 tubes contenant 1 plantule de *T. pratense*, avec charbon de bois, inoculés au moyen de la souche S. 22, spécifique des *Medicago* et incapable de provoquer la nodulation sur *Trifolium*,
- 12 tubes contenant 1 plantule de *T. pratense*, sans charbon de bois, et inoculés au moyen de la souche S. 22,
- 12 tubes contenant 1 plantule de *T. pratense*, sans charbon de bois, et inoculés au moyen de la souche S. 50,
- 12 tubes contenant 1 plantule de *T. pratense*, sans charbon de bois, non inoculés.

Observations:

1. Toutes les plantules en tubes, avec charbon de bois, ont un développement plus rapide et plus abondant. Le fait est très net, même pour les plantes non inoculées. Le charbon de bois, par sa teneur

en nitrate notamment, fournit à la plante des éléments qui permettent un développement meilleur.

2. Les plantes avec charbon de bois, inoculées au moyen de la souche spécifique S.50, forment beaucoup plus de nodules que les plantes inoculées sans charbon de bois.

Ces nodules, beaucoup plus nombreux, souvent très rapprochés, confirment les résultats obtenus par NUTMAN (19).

Si les nodules sont plus nombreux, ils sont également plus petits. Contrairement aux auteurs précités, nous ne pouvons prétendre que la fixation d'azote soit augmentée. Sans doute, les plantes inoculées sans charbon de bois sont moins belles; mais les plantes non inoculées avec charbon de bois ont un développement nettement accru, qui peut être dû à la présence de nitrate dans le charbon de bois.

3. Fait important, sur certaines plantules avec charbon de bois, inoculées au moyen d'une souche non spécifique pour *Trifolium*, mais spécifique de *Medicago*, nous avons observé, après 1 mois de culture la présence de formations anormales sur les racines. Il s'agit, dans tous les cas, de "gonflements" très nettement marqués des racines. Il ne s'agit pas de nodules au sens strict; en effet ceux-ci sont, sur *Trifolium*, distincts de la racine sur laquelle ils se forment. Dans le cas qui nous occupe, c'est la racine toute entière qui est localement "gonflée". Microscopiquement, ces formations sont simplement constituées de la multiplication des cellules du parenchyme cortical. En volume, elles atteignent la dimension de petits nodules.

Il est remarquable que ces formations contiennent à l'état pur le *Rhizobium* qui a servi à l'inoculation. En effet, à partir de ces formations désinfectées extérieurement, nous avons pu, dans chaque cas, réisoler des bactéries, macroscopiquement et microscopiquement indiscernables de la souche S. 22 qui a servi à l'inoculation. Cette souche S. 22, possède une spécificité antigénique marquée. Pour démontrer avec plus de précision encore que les isollements réalisés à partir des formations anormales contiennent bien le *Rhizobium* S. 22, nous avons vérifié antigéniquement leur identité. A partir du sérum S. 22 dont nous disposions, nous avons obtenu des agglutinations très nettes, jusqu'à la dilution 1 : 20.000; les réactions d'agglutinations obtenues avec les souches réisolées correspondaient exactement à celles obtenues avec la souche S. 22.

Ajoutons que les souches ainsi réisolées, spécifiques à l'origine de *Medicago*, ont été, après réisolement de *Trifolium*, inoculées à

de jeunes plantules de *Medicago sativa*, avec un résultat positif dans tous les cas. Nous avons obtenu des nodules "effectifs", de la même façon que par inoculation avec la souche S. 22. Il importe de signaler que les formations anormales ont été obtenues sur une partie seulement des plantules (6 tubes sur 12 ont réagi).

De plus, si les nodules obtenus au moyen d'une souche spécifique se forment sur *Trifolium*, assez régulièrement dès le 12^{me} jour après l'inoculation, les formations dont nous parlons apparaissent irrégulièrement, au bout d'un temps plus ou moins long, variable d'un tube à l'autre (1 mois au moins).

Dans aucun cas, nous n'avons pu observer sur ces formations, le moindre signe d'"effectivité", décelable à la couleur des tissus.

Ainsi donc, sur des plantules de *Trifolium*, cultivées aseptiquement en présence de charbon de bois et inoculées avec une souche spécifique des *Medicago*, nous avons obtenu des formations radiculaires anormales, contenant la bactérie qui peut en être réisolée.

Microscopiquement, macroscopiquement et antigéniquement, ces isolats correspondent à la souche S. 22 qui a servi à l'inoculation. Ces souches réisolées de *Trifolium* donnent toujours des nodules "effectifs" normaux sur *Medicago*.

Dans un deuxième essai:

La même expérience a été répétée sur *Medicago sativa*, en conditions aseptiques également. Les tubes suivants ont été préparés:

- 12 tubes contenant une plantule de *Medicago sativa*, avec charbon de bois, non inoculés.
- 12 tubes contenant une plantule de *Medicago*, avec charbon de bois, inoculés au moyen de 1 cc d'une suspension aqueuse de la souche S. 22, spécifique des *Medicago*,
- 12 tubes contenant une plantule de *Medicago*, avec charbon de bois, inoculés au moyen de la souche S. 50, spécifique des *Trifolium*, mais incapable de provoquer la nodulation sur *Medicago*,
- 12 tubes contenant une plantule de *Medicago*, sans charbon de bois, inoculés au moyen de la souche S. 50,
- 12 tubes contenant une plantule de *Medicago*, sans charbon de bois, inoculés au moyen de la souche S. 22,
- 12 tubes contenant une plantule de *Medicago*, sans charbon de bois, non inoculés.

Observations:

1. Au sujet du développement des plantules, les mêmes consta-

tations ont été faites sur *Medicago sativa* que sur *Trifolium pratense*. Le développement fut nettement, pour toutes les plantules, plus rapide et plus abondant, en présence de charbon de bois.

2. Les plantes de *Medicago*, inoculées au moyen de la souche spécifique S. 22, en présence de charbon de bois, forment des nodules beaucoup plus nombreux, mais généralement nettement plus petits. Ici encore, la production par les racines de substances inhibitrices constitue une hypothèse plausible, qui semble bien se vérifier.

3. Dans 4 tubes sur 12, inoculés au moyen de la souche S. 50, en présence de charbon de bois, nous avons obtenu, 6 semaines environ après l'inoculation, des formations anormales particulières.

Il ne s'agit pas ici, comme dans le cas des *Trifolium*, de "gonflements" de la racine, assez nombreux. Il s'agit de la formation, pour tout un système racinaire, de 1 ou 2 très grosses proliférations, pouvant atteindre 2 cm. La surface de ces formations est irrégulière, et apparaît un peu comme une grappe, suspendue à la radicelle.

Exactement comme dans le cas des *Trifolium*, ces néoformations contiennent une bactérie, qui a pu être réisolée et qui est exactement identifiable, comme dans le cas précédent, avec la souche qui a servi à l'inoculation.

Ici encore, la souche réisolée donne des nodules normaux, "effectifs" sur *Trifolium pratense*.

En aucun cas, les néoformations obtenues sur *Medicago* ne montrent le moindre signe d'"effectivité".

Ainsi donc, sur des plantules de *Medicago*, cultivées aseptiquement en présence de charbon de bois et inoculées avec une souche spécifique des *Trifolium*, nous avons obtenu des formations racinaires anormales, contenant la bactérie qui peut en être réisolée.

Dans un troisième essai:

Dans l'introduction du présent article, nous avons rappelé l'impossibilité, dans les conditions où nous travaillons, d'obtenir des nodules sur *Trifolium ambiguum*, même au moyen d'une souche spécifique des *Trifolium*. Nous avons signalé que, en greffant sur *T. ambiguum* des greffons de *T. pratense* et *T. repens*, nous avons obtenu des nodules sur *T. ambiguum* mis en présence de la souche S. 50, spécifique des *Trifolium*.

Il faut souligner que, jamais, le moindre signe d'"effectivité" ne s'est révélé, sur ces nodules obtenus après greffes.

Nous avons donc imaginé d'inoculer, avec la souche S. 50, en

culture aseptique, des plantules de *T. ambiguum*, se développant en présence de charbon de bois.

- 12 tubes ont été garnis d'une plantule de *T. ambiguum*, non inoculée,
- 12 tubes ont été garnis d'une plantule de *T. ambiguum*, non inoculée, en présence de charbon de bois,
- 12 tubes ont été garnis d'une plantule de *T. ambiguum*, en présence de charbon de bois et inoculée au moyen de la souche S. 50, spécifique de *Trifolium* mais normalement sans effet sur *T. ambiguum*,
- 12 tubes ont été garnis d'une plantule de *T. ambiguum*, sans charbon de bois, inoculée au moyen de la souche S. 50.

Observations:

1. Pour cette troisième espèce, *Trifolium ambiguum*, nous avons également observé un développement plus rapide et plus abondant, dû au charbon de bois.

2. Les plantules inoculées de S. 50, sans charbon de bois, n'ont jamais montré le moindre nodule.

3. 3 plantules sur 12, inoculées de S. 50, en présence de charbon de bois, ont montré des nodules.

4. De plus, les nodules observés ici étaient "effectifs", à en juger à leur couleur rouge intense, transparaissant à l'extérieur.

Ainsi, en présence de charbon de bois, des plantules de *T. ambiguum* inoculées de la souche S. 50, spécifique des *Trifolium*, portent des nodules, alors qu'elles n'en portent jamais, sans charbon de bois. De plus, ces nodules sont "effectifs", alors que les nodules obtenus par la méthode des greffes n'ont jamais montré le moindre pouvoir fixateur d'azote.

Signalons d'ailleurs que, à notre connaissance, aucun nodule "effectif" n'avait pu être observé, jusqu'à présent, sur *Trifolium ambiguum*. C'est ainsi que PARKER et ALLEN (23) ont inoculé à des *T. ambiguum* 35 souches de *Rhizobium*, originaires de 10 espèces différentes de *Trifolium*, sans jamais relever la moindre trace d'"effectivité" sur cette espèce.

Dans un quatrième essai:

Etant donné les relations étroites qui lient le genre *Rhizobium*, avec l'espèce *A. tumefaciens*, agent du crown-gall, nous avons inoculé des plantules de *Medicago sativa*, cultivées aseptiquement en présence de charbon de bois, au moyen de 1 cc d'une suspension aqueuse d'une souche de *A. tumefaciens*.

On sait que ce microorganisme provoque, après blessure préalable, la formation de tumeurs au collet et sur la tige de nombreuses espèces végétales ; de ce fait, cette bactérie ne montre aucune spécificité comparable à celle des souches de *Rhizobium*. La blessure est toujours apparue comme indispensable à l'éclosion de ces néoformations. KLEIN (12) d'ailleurs, apporte des faits à l'appui de ce point de vue.

Or, sans blessure préalable, nous avons obtenu sur une radicelle de 2 plantules de *Medicago* (parmi 12 répétitions), inoculées de *A. tumefaciens* en présence de charbon de bois, la formation d'une galle arrondie, d'aspect ridé, qui, macroscopiquement, ne se différencie pas d'une tumeur obtenue sur tige, par blessure préalable.

Notons, dans ce cas, comme dans les précédents, la réaction irrégulière des plantules (2 cas sur 12 dans cet essai).

Conclusion de ces essais avec charbon de bois.

L'adjonction de charbon de bois au milieu sur lequel sont cultivées deux espèces Légumineuses, *Trifolium pratense* et *Medicago sativa*, permet à des souches de *Rhizobium* non spécifiques, d'envahir le système racinaire, pour y former des excroissances anormales, d'où la bactérie en cause peut être réisolée.

Tout se passe comme si le charbon de bois adsorbait des substances, sécrétées par les racines, substances qui empêchent l'invasion des racines par des souches non spécifiques.

De plus, le charbon de bois rend spécifique et "effective", une souche de *Rhizobium*, normalement inactive sur *T. ambiguum*.

Ces faits, outre leur intérêt en ce qui concerne les phénomènes de spécificité, semblent bien confirmer la théorie de NUTMAN (16), de THORNTON (23), de LUDWIG et ALLISON (13) sur l'excrétion, par les racines de Légumineuses, de substances stimulantes et inhibitrices, qui régularisent la formation et l'"effectivité" des nodules de Légumineuses.

En ce qui concerne les propriétés particulières du charbon de bois, il convient de signaler ici certaines constatations faites dans les conditions naturelles. ANDERSON et SPENCER (2) ont observé sur des parcelles de *Trifolium* généralement dépourvues de nodules actifs, établies en Australie, "a few small, isolated patches of normal, dark green clover, invariably well nodulated, occurred on the trials. These patches were erratically distributed and were not associated with any specific treatment. An obvious concentration of charcoal

was often found on the grounds at these patches". Selon HELY BERGERSEN et BROCKWELL (10) il est connu que l'établissement du trèfle, sur des sols donnés, s'effectue très rapidement aux emplacements de feux de bois. Au Congo belge, PH. CULOT (communication personnelle) a noté que, dans une parcelle entière de *Pueraria javanica* Benth, les seuls beaux nodules ont été trouvés à un endroit où il y avait accumulation de charbon de bois.

OBSERVATIONS COMPLÉMENTAIRES.

Si les néoformations radiculaires obtenues sur racines de *Trifolium* et de *Medicago*, au moyen de souches non spécifiques, en présence de charbon de bois, interviennent réellement dans les processus naturels d'adaptation à des Légumineuses diverses de souches quelconques de *Rhizobium*, elles devraient pouvoir être observées dans la nature.

Or, jusqu'à ces derniers temps, nous n'avons jamais pu faire d'observations qui puissent éclairer le problème.

Nous avons eu l'occasion, récemment¹⁾, de multiplier en Afrique équatoriale belge, nos observations sur l'état naturel, au point de vue nodulation, de diverses Légumineuses.

Au cours des nombreuses prospections réalisées, nous avons rencontré, sur diverses espèces que nous allons citer, des formes caractéristiques, semblables à celles observées dans les conditions artificielles, rapportées ci-dessus.

Sur *Desmodium adscendens*, *Mimosa invisa*, *Pueraria javanica*, *Phaseolus angularis*, et surtout sur *Canavalia ensiformis*, de nombreuses excroissances caractéristiques ont été observées, bien souvent, sur des racines portant également de vraies nodosités. Ces excroissances, comme celles observées "in vitro" sur *Trifolium*, en présence de charbon de bois, sont constituées par des "gonflements" de la racine, tout à fait typiques.

Fait significatif, nous avons pu, dans nombre de cas, isoler de ces excroissances, après un sévère traitement de désinfection externe, des souches bactériennes, macroscopiquement et microscopiquement indifférenciables des *Rhizobium*. Ces souches présentent notamment de nombreuses formes en Y, en X, en T, si caractéristiques des bactéries de la famille des *Rhizobiaceae*.

¹⁾ Ces observations ont été rendues possibles, grâce à l'aide de l'Institut National pour l'Etude Agronomique du Congo Belge. Nous tenons à remercier vivement la Direction de cet Institut.

Ces néoformations observées ne peuvent être des nodosités en formation. En effet, sur des espèces où les nodosités sont nettement distinctes des racines sur lesquelles elles se forment, les excroissances en cause sont constituées de masses tissulaires qui intéressent la racine, modifiée dans son diamètre. Elles diffèrent donc essentiellement des nodosités jeunes en formation, dont il est aisé de suivre le développement; dès leur formation, celles-ci présentent un aspect particulier pour chaque espèce végétale, aspect que l'on retrouve sur les nodosités formées.

Il ne peut s'agir non plus des "faux nodules", au sens de ALLEN et ALLEN (1), faux nodules dont on n'a jamais pu isoler un micro-organisme.

Ces excroissances ne peuvent être confondues avec des galles dues aux nématodes, qui sont fréquentes en conditions équatoriales, mais qui contiennent l'anguillule parasite, très caractéristique.

Nous n'avons pas manqué de faire le rapprochement entre ces "nodosités", que nous appellerons intermédiaires, et celles observées, "in vitro", au cours des essais d'adaptation en présence de charbon de bois.

Conclusions générales.

A la suite des observations, dans les conditions naturelles, effectuées en Afrique Centrale d'une part, et étant donné les résultats obtenus lors de nos essais d'adaptation de souches non spécifiques en présence de charbon de bois, d'autre part, nous formulons l'hypothèse que les formes anormales de nodosités observées, constituent des formes "intermédiaires", témoins d'un processus d'adaptation de souches quelconques de *Rhizobium* à des Légumineuses déterminées.

L'excrétion par les racines de Légumineuses de substances stimulantes et inhibitrices de la nodulation semble ainsi se confirmer. En adsorbant ces substances, nous sommes parvenus à détruire l'équilibre qu'exercent ces substances sur les phénomènes de croissance en général et plus particulièrement sur la formation des nodules et leur comportement ultérieur.

Des essais seront poursuivis, qui concernent notamment l'inoculation, en conditions aseptiques, des souches isolées de ces "excroissances" aux espèces Légumineuses en cause.

L'évolution des races bactériennes, réisolées chaque fois, pourra ainsi être suivie.

Le sol possède des propriétés d'adsorption vis à vis des substances secrétées par les racines, propriétés qui pourraient expliquer le phénomène d'adaptation de souches de *Rhizobium*, que l'on observe dans la nature.

Si l'hypothèse que nous formulons se vérifiait par la suite, elle expliquerait facilement toutes les observations contradictoires qui ont été effectuées par différents auteurs, sur la spécificité de l'hôte.

Les *Rhizobium* seraient bien à considérer comme un vaste groupe de bactéries, possédant des caractères communs et capables – dans certaines conditions – de former des nodules sur les racines de Légumineuses, en fixant ou en ne fixant pas l'azote atmosphérique.

Ce groupe serait donc constitué de formes en perpétuelle évolution, évolution liée à l'influence de facteurs internes et externes (sol, plantes, conditions climatiques).

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TORULOPSIS APICOLA NOV. SPEC., NEW ISOLATES FROM BEES

by

M. HAJSIG

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When examining the normal fungal flora in the intestinal tract of bees, we isolated 7 strains of one yeast species from 6 out of 8 beehives. From each hive 10 bees were examined. These strains belonged to the genus *Torulopsis*, as given by LODDER and KREGER-VAN RIJ (1952), but not to any known species. We are therefore describing a new species, for which we propose the name *Torulopsis apicola*.

The description of this new budding yeast is based on the standard methods used by LODDER and KREGER-VAN RIJ (1952) and by WICKERHAM (1951) for the identification of yeasts.

Torulopsis apicola nov. spec.

Latin description:

In musto maltato cellulae ovideae, subovideae aut rotundae, singulae, binae, catenatae, in racemis aut stellis parvis, $(2,2-4) \times (3-4,3) \mu$. Sedimentum annulusque formantur.

In agar maltato formae et dimensiones cellularum eadem sunt quae in musto maltato. Cultura (post unum mensem, 17°C.) mollis, parum nitens, glabra, flavalbida, colliforma, margine glabro.

Pseudomycelium nullum observatum est.

Fermentatio (sera) glucosi et sucrosi. Glucosum, sucrosum et raffinsum assimilantur. In medio minerali cum alcohole aethylico non crescit. Nitraskalicus non assimilatur. Arbutinum non finditur. Vitamina extranea sunt necessaria ad crescentiam.

Non crescit sub 37°C.

Isolata ex apibus.

Growth in malt extract: After 3 days at 25°C., the cells are small, oval, sub-oval to round, single, in pairs, in small chains, clusters or star-like formations. Cell size $(2.2-4) \times (3-4.3) \mu$ (see Fig. 1). A sediment and a thin ring are formed. After one month ring and sediment are maintained but no pellicle has developed.

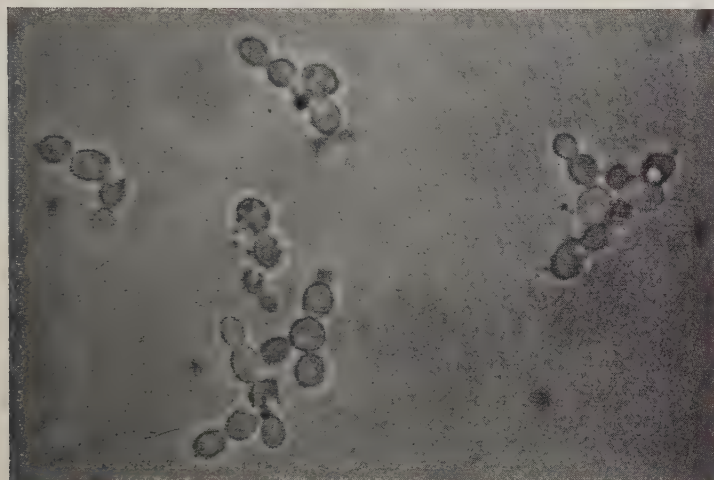


Fig. 1. *Torulopsis apicola*. After 3 days in malt extract (1500 \times).

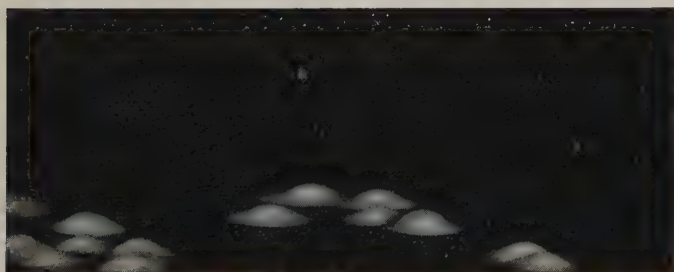


Fig. 2. Colonies of *Torulopsis apicola* on malt agar after 14 days.
(Photographs: J. SCHUUR, Delft)

Growth on malt agar: After 3 days at 25°C., the cells are of the same size as those in malt extract. Small chains, clusters or star-like formations are seldom found. Colonies (after one month at 17°C.) are rounded-conical, soft, glistening and smooth, cream coloured; margin smooth (see Fig. 2). The streak culture (after one

month at 17°C.) is well-grown, brownish-white, flat, soft, smooth and glistening.

Slide culture: Pseudomycelium was not present after 14 days.

Sporulation was not observed on malt agar (after one month), nor on the usual sporulation media.

Fermentation of glucose and sucrose occurs though retarded. Galactose, maltose, lactose, raffinose and inulin are not fermented. Fermentation of glucose begins after 4-5 days, of sucrose after 4-10 days (in most strains after 7 days).

Sugar assimilation: Glucose, sucrose and raffinose are assimilated. Galactose, maltose and lactose are not assimilated.

Assimilation of potassium nitrate: Absent.

Splitting of arbutin: Absent.

Ethanol as sole source of carbon: No growth.

Growth on vitamin-free medium: Absent.

Acid production on chalk agar: Observed.

Growth on concentrated sugar medium: Fair development.

Growth at 37°C.: Absent.

DISCUSSION.

The inclusion of the new strains in *Torulopsis* is based on the description of this genus by LODDER and KREGER-VAN RIJ (1952). The species seems to have many characteristics in common with *T. bacillaris* and *T. stellata*. Therefore, these species together with *T. apis* (LAVIE, 1954) have been studied along with the new isolates discussed in this note.

The size of the cells of the isolated strains is smaller than that of either *T. bacillaris*, *T. stellata* or *T. apis* (see Table 1). Other differences exist regarding the form of the colonies (rounded-conical in our strains, but flat in the known species) and also regarding growth in malt extract. *T. bacillaris* and *T. stellata* formed only sediment whereas our isolates formed a ring and a sediment; though *T. apis* formed islets after 1 month. The growth of the new isolates on streak cultures is greater than that of the other species.

There is a striking difference in the lag period of fermentation, the new isolates, in contrast with *T. bacillaris* and *T. stellata*, having delayed fermentation of glucose and sucrose and being unable to ferment raffinose. *T. apis* does not ferment sugars.

To judge the rate of acid production the strains were cultivated on CUSTERS' medium ¹⁾. They produced so much acid that after 10 days at 25°C. the calcium carbonate had almost entirely disappeared. For *T. apis*, acid production was not observed even after 10 days; and *T. bacillaris* and *T. stellata* produced only small quantities of acid.

Our isolates grew rapidly (except 1 strain) on a concentrated sugar medium ²⁾, whereas *T. apis* grew only slowly after 1 week.

Besides these described species, *T. bacillaris*, *T. stellata* and *T. apis*, we have also compared our strains with an undescribed strain, isolated in 1953 from the tracheae of a bee, which was sent by Dr J. GUILHON (École vétérinaire d'Alfort, France) to the Centraal-bureau voor Schimmelcultures in Delft. This yeast, provisionally identified as a variety of *T. bacillaris*, proved to be identical in all properties with our strains.

TABLE 1.

	<i>T. apicola</i>	Strain of J. GUILHON	<i>T. bacil- laris</i>	<i>T. stellata</i>	<i>T. apis</i>
Size of the cells	(2.2-4) × (3-4.3) μ	(2.2-3.5) × (3.5-4.2) μ	(2-4) × (3.5-5.5) μ	(3.5-4.5) × (3.8-5.2) μ	(1.8-4) × (3-5.5) μ
Growth-formation on malt extract	Ring and sediment	Ring and sediment	Only sediment	Only sediment	Ring, sediment and islets
Fermentation	gluc. +* sucr. +* raff. —	+ ¹⁾ + ¹⁾ — ¹⁾	+	+	—
Acid production	+	+	weak	weak	—
Growth on conc. sugar medium	+, seldom—	+	+	+	weak

* late.

Because of significant morphological and biochemical differences from known species of *Torulopsis*, our strains as well as that of J. GUILHON must be regarded as representatives of a new species for which we propose the name *Torulopsis apicola*.

The cultures have been deposited in the Yeast Division of the

¹⁾ Custers' medium: yeast extract agar with 5% glucose and 0.5% calcium carbonate.

²⁾ Concentrated sugar medium: 60% (w/w) glucose in yeast agar.

Centraalbureau voor Schimmelcultures at Delft (Holland) and in the Institute for Microbiology and Infectious Diseases of the Veterinary Faculty, University of Zagreb (Yugoslavia). Our strain Nr. III-7 has been designated as the type culture of the species *Torulopsis apicola*.

Acknowledgements.

This study has been made at the Yeast Division of the Centraalbureau voor Schimmelcultures at Delft (Holland). We thank Prof. T. O. WIKÉN for hospitality and the assistants of the Yeast Division, especially Dra W. CH. SLOOFF, for their interest and critical advice. We thank Prof. I. TOMAŠEC, chief of the Institute for Biology and Pathology of Bees (Veterinary Faculty at Zagreb, Yugoslavia) for the bees, from which these isolates were obtained. Further we wish to express our thanks to Mr. J. SCHUUR for kindly preparing the photographs.

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(From the Bacteriological Department, State Institute of Public Health,
Oslo, Norway).

OCCURRENCE OF YEASTS IN THE INTESTINAL TRACT OF 20 PATIENTS DURING TREATMENT WITH BACITRACIN AND NEOMYCIN¹⁾

by

SIGNY REIERSÖL

(Received September 11, 1957).

Twenty patients at Ullevål Hospital, Oslo, who were to undergo operations on colon were orally treated with 3 g bacitracin and 3 g neomycinsulphat during three days daily. This treatment showed sufficient preoperative bacteriological reduction of the intestinal tract, while the occurrence of yeasts increased. HERTZBERG and LOE (1956) have published the results of the clinical and bacteriological findings of this investigation in a previous paper. The present paper shows the results of the mycological investigation.

METZGER, WRIGHT and DiLORENZO (1954) who investigated 137 stool samples from 17 patients found no relationship between gastrointestinal distress and the numbers of yeasts in the stool. They point out that a greater percentage of yeasts would be recovered when the bacterial population had been reduced by drugs and this may lead to an erroneous diagnosis of moniliasis. However, as stated by HARRIS (1950), PAINE (1952) and ZIMMERMAN (1950) the antibiotics cause an alteration of the intestinal flora to such extent that a B-complex vitamin-deficiency results, and this may predispose to mucocutaneous moniliasis.

Most of the authors who have studied intestinal yeasts as a sequel to antibiotic therapy have studied all yeasts together without regarding the separate yeast species. Besides the alteration in numbers of yeasts, it should also be of interest to see which of the yeast species occur in the intestine and which of them are most frequent during different circumstances.

¹⁾ The technical assistance of Miss ANNA HELGERUD is gratefully acknowledged.

METHOD.

Fresh stool was weighed and serially diluted with sterile sodium chloride solution. The dilutions were plated with Sabouraud glucose agar containing 100 μ g of streptomycin per milliliter of medium. After incubation at 25°C. for three to six days yeast cells were counted directly from the incubated plates. Subcultures were made from these plates for further examination. The dilutions of the first four stool samples were also plated with Litman oxgall agar containing the same per cent of streptomycin. This medium gave no more growth of yeasts than Sabouraud's agar which is much simpler to prepare.

RESULTS.

Yeasts occurred in 7 of the 20 patients – 37% – before treatment and in 18 patients – 90% – after treatment. As shown in table 1 the yeasts have also increased in numbers in the seven patients where yeasts also occurred in the intestinal tract before treatment. Only in 3 cases growth of yeasts was completely lacking. In 11 cases yeast growth set in during or after treatment. Table 2 shows the yeast species occurring in the stool samples before and after treatment. *Candida albicans* occurred in 15 stool samples – 5 before treatment and 10 after treatment. *Candida mycoderma* occurred in 6 stool samples – 2 before treatment and 4 after treatment. In one patient (No. 19, table 1) there was growth of *C. mycoderma* before treatment and of *C. albicans* after treatment. *Candida krusei* and *Candida tropicalis* both occurred in 2 stool samples. The other 5 yeast strains isolated occurred each in one stool sample. They were as follows: *Candida parapsilosis*, *Torulopsis glabrata*, *Rhodotorula mucilaginosa*, *Rhodotorula minuta*, and *Pichia membranaefaciens*.

DISCUSSION.

The present investigation supports the results of other investigators concerning the increased yeast growth in the intestinal tract in patients treated with antibiotics (METZGER *et al.*, 1954; HARRIS, 1950; ZIMMERMAN, 1950; PAINE, 1952; PAPPENFORT *et al.*, 1951; HINDISCH *et al.*, 1955). It should have been interesting to see the yeast population in the intestine of the same patients a

TABLE 1.

Influence of oral therapy with bacitracin and neomycin on numbers of yeasts recovered from intestinal tract of patients.

Case	Yeast counts before treatment (number of cells per gram wet stool)	Yeast counts after treatment
1	0	500 <i>Candida parapsilosis</i>
2	0	88,000 <i>Candida albicans</i>
3	250 <i>Candida albicans</i>	120,000 <i>Candida albicans</i>
4	0	1,600,000 <i>Candida albicans</i>
5	0	42,000 <i>Candida mycoderma</i>
6	20,000 <i>Candida albicans</i>	1,200,000 <i>Candida albicans</i>
7	0	500 a. <i>Candida tropicalis</i> b. <i>Candida mycoderma</i>
8	0	900 <i>Torulopsis glabrata</i>
9	0	400 <i>Pichia membranefaciens</i>
10	600 <i>Candida albicans</i>	260,000 <i>Candida albicans</i>
11	0	0
12	60 a. <i>Candida mycoderma</i> b. <i>Rhodotorula mucilaginosa</i>	9,000 <i>Candida mycoderma</i>
13	22,000 a. <i>Candida albicans</i> b. <i>Rhodotorula minuta</i>	115,000 <i>Candida albicans</i>
14	0	0
15	0	342,000 <i>Candida albicans</i>
16	0	16,000 a. <i>Candida tropicalis</i> b. <i>Candida krusei</i>
17	45,000 <i>Candida albicans</i>	1,340,000 <i>Candida albicans</i>
18	0	11,000 a. <i>Candida krusei</i> b. <i>Candida mycoderma</i>
19	200 <i>Candida mycoderma</i>	100,000 <i>Candida albicans</i>
20	0	185,000 <i>Candida albicans</i>

TABLE 2.

Frequency of yeast species during oral treatment with bacitracin and neomycin.

	Before treatment	After treatment
<i>Candida albicans</i>	5	10
<i>Candida mycoderma</i>	2	4
<i>Candida krusei</i>	0	2
<i>Candida tropicalis</i>	0	2
<i>Candida parapsilosis</i>	0	1
<i>Torulopsis glabrata</i>	0	1
<i>Rhodotorula mucilaginosa</i>	1	0
<i>Rhodotorula minuta</i>	1	0
<i>Pichia membranefaciens</i>	0	1

sufficiently long time after treatment to get a "normal" stool. We regret that such material was not available. However, according to other investigators, the intestinal yeast population of normal persons varies widely from one week to another in the same persons (METZGER *et al.*, 1954; WINDISCH *et al.*, 1955).

Regarding the occurrence of the separate yeast species *Candida albicans* is by far the most frequent. It occurs in 25% of the stool samples of the patients before treatment and in 50% after treatment. This is in good agreement with the investigation of WINDISCH and STAIB (1955). Besides there is a remarkably high frequency of *Candida mycoderma*. From one patient there was isolated *Pichia membranefaciens*, which is regarded as the perfect form of *Candida mycoderma*. WINDISCH and STAIB (1955) who investigated stool samples from 1100 patients during a year, found that *C. mycoderma* occurred every month during the whole year though not so frequently as *C. krusei*. As shown in Table 1 *Rhodotorula* species has been isolated from two patients (No. 12 and No. 13) before treatment: *Rh. mucilaginosa* together with *C. mycoderma* and *Rh. minuta* together with *C. albicans*. After treatment there was increasing growth of *C. mycoderma* and *C. albicans* respectively, while the *Rhodotorula* species did not occur at all. Probably the increasing growth of the *Candida* species has suppressed the growth of the red yeasts.

S u m m a r y.

A mycologic investigation of stool samples of 20 patients treated with bacitracin and neomycin shows increasing growth of yeasts during the therapy. Thus 90% of the patients had yeasts in the stools after treatment, while 37% before treatment. Most of the isolated yeast species are *Candida* species.

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(From the Bacteriological Department, State Institute of Public Health, Oslo, Norway and from the Department of Microbiology, University of Otago, Dunedin, New Zealand).

A NEW *CRYPTOCOCCUS* SPECIES

by

SIGNY REIERSÖL and **MARGARET DI MENNA**

(Received September 11, 1957).

Searching for fungi in gastric lavages from tuberculous patients in Norway there was found a yeast strain, V 39, that turned out to be a new *Cryptococcus* species. Because of its origin from a gastric lavage it was called *Cryptococcus gastricus*. In liquid media the growth of this species is rather poor. On solid media the growth is slow, but after several days cultivation the growth may be good especially on wort agar. The size of the colonies varies a good deal, after 12 days on a petri dish with wort agar (spread through water) the colonies may vary from barely visible to 6 mm in diameter.

Standard description of *Cryptococcus gastricus*:

Growth in malt extract: After 3 days at 25°C. cells are round-oval to long-oval $(3.5 - 6) \times (5.5 - 11)\mu$, single or in pairs.

After one month at 17°C. a thin ring and a sediment are formed.

Growth on malt agar: After 3 days at 25°C. cells have the same shape and size as in malt extract.

The cells are surrounded by a capsule. "Starch" is produced.

The streak culture after one month at 17°C. is yellowish-brown, slightly wrinkled in the middle with a smooth and lobated margin.

The culture may be mucous.

Slide cultures: Some elongate cells are formed, but no pseudomycelium.

Fermentation: Absent.

Sugar assimilation:	Glucose	+	Maltose	+
	Galactose	+	Lactose	—
	Saccharose	—		

Assimilation of potassium nitrate: Absent.
Ethanol as sole source of carbon: No growth.
Splitting of arbutin: Negative.

Cryptococcus gastricus nov. spec.

In musto maltato cellulae subovoideae aut longoovoideae $(3.5-6) \times (5.5-11) \mu$, singulae aut binae. Sedimentum et anulus subtilis formantur. In agar maltato formae et dimensiones cellularum eadem sunt quae in musto maltato. In medium liquidis agarinisque cellulae capsula circumdatae sunt. Amylum formatur. Cultura (post unum mensem, $17^{\circ}\text{C}.$) flavifusca, in parte media parum crispulata, margine undulato et glabro, in parte interdum mucosa. Pseudomycelium nullum. Fermentatio nulla. In medio minerali cum glucoso, galactoso, maltoso crescit. Nitras calicus non assimilatur. In medio minerali cum alcohole aetylico non crescit.

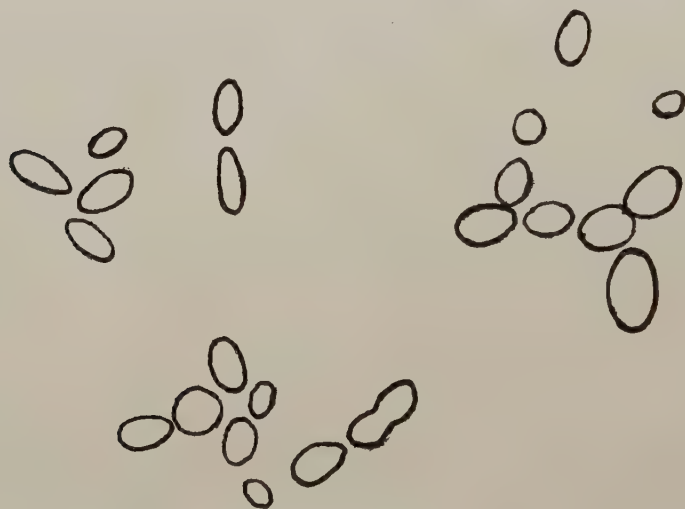


Fig. 1. *Cryptococcus gastricus*. After 3 days in malt extract. $1000 \times$.

DISCUSSION.

After 6 - 8 weeks cultivation on glucose-agar and on Gorodkova agar some cells formed one or more sacklike protuberances filled with a granular substance which sometimes seemed to be spores. Staining after Ziehl-Nielsen's method these structures were acid-fast. Because of the close resemblance of this yeast to the genus *Lipomyces* described in LODDER and KREGER-VAN RIJ's taxonomy, one of us (REIERSÖL) working with the strain at first

thought it might be a new *Lipomyces* species. She sent it to the Centraalbureau voor Schimmelcultures, Yeast Division, Delft. Dr W. SLOOFF examined the strain and wrote among other things in a return-letter: "Because you mentioned acid-fast structures in your letter of January 2, I made preparations after Ziehl-Nielsen's method and could confirm your observations. In many cells the contents showed the acid-fast stain, often also a granulated texture. Spores, however, I have not observed. It has been mentioned frequently in literature that several yeasts produce "chlamydospores": older cells, in most cases provided with a thick wall or capsule. LANGERON and LUTERAAN (Ann. parasitol. humaine et comparée 22, 254, 1947 and Compt. rend. 229, 382, 1949) mentioned that chlamydospores are characterized by a high degree of acid-

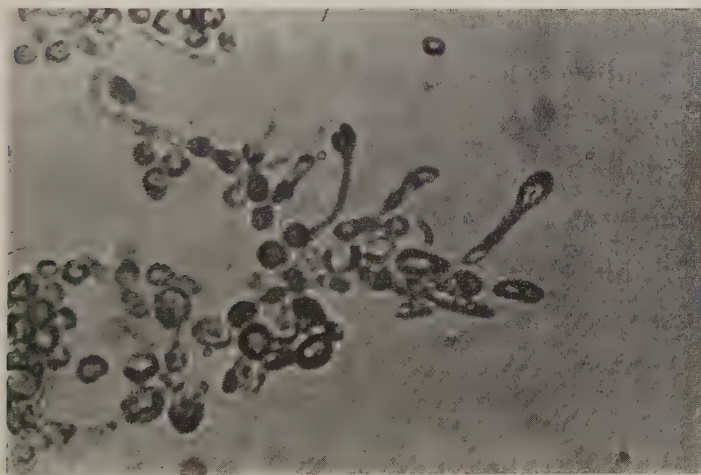


Fig. 2. *Cryptococcus gastricus*. Slide culture. Potato agar.

fastness. It seems only reasonable to consider the acid-fast structures in V 39 as chlamydospores.

As this yeast produces a very clear "starch" reaction on Ashner's agar (after 3 weeks) there will be no objections against its comprising into the genus *Cryptococcus*.

In this genus V 39, according to its assimilation pattern, represents a new species.

To avoid confusion it must be stressed that in *Lipomyces* we possess no strain which could produce a clear cut "starch" reaction

as is normally encountered in *Cryptococcus*. WICKERHAM (Ann. Rev. Microbiol. 6, 317, 1952) states to have observed "starch" production in *Lipomyces* in shake cultures. We also have found a green coloration in some *Lipomyces* strains, in contrast with the dark blue reaction in *Cryptococcus*."

The same species was earlier isolated in New-Zeeland by one of the authors (DI MENNA) from a soil near Dunedin. She recovered a total of six strains of the species.

In 1955 BENHAM searched for the perfect stage of *Cryptococcus neoformans*. After 2 months' cultivation on malt extract agar she found sac-like cells within some of which were 2, 4 and 8 spores. She therefore thought that *Cryptococcus neoformans* and *Lipomyces starkey* might be one and the same organism.

In studying the genus *Cryptococcus* BENHAM in 1956 discusses the same phenomenon and points out the close relationship between *Cryptococcus neoformans* and the genus *Lipomyces*.

However, as quoted above the Centraalbureau in Delft possesses no strain of *Lipomyces* which could produce a clear cut "starch" reaction.

S u m m a r y.

A new *Cryptococcus* species, *Cryptococcus gastricus*, is described. Its relationship to the genus *Lipomyces* is discussed.

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(National Institute of Public Health, Utrecht, The Netherlands).

A NEW *SALMONELLA* TYPE (*S. QUIMBAMBA*)

by

A. CLARENBURG, E. H. KAMPELMACHER and
L. M. VAN NOORLE JANSEN

(Received February 4, 1958).

In May 1957 a new *Salmonella* type was isolated by Dr D. A. A. MOSSEL (Central Institute for Nutritional Research, Utrecht) from fish-meal of Portuguese Angola origin.

The culture had the following biochemical behaviour: No fermentation of adonitol, inositol, lactose, salicin, sucrose and glycerol. No production of indole, no decomposition of urea and no liquefaction of gelatin. Rapid fermentation of arabinose, dulcitol, glucose (with gas), maltose, mannitol, rhamnose, sorbitol, trehalose and xylose. Positive reaction in Stern's glycerol-fuchsin broth, and formation of H_2S . Positive reaction in d-tartrate and mucate after 1 day, and negative reaction in l- and i-tartrate after 14 days. Nitrates were reduced, the Voges Proskauer reaction was negative and the methyl red reaction was positive. The culture did not grow in KCN substrate.

On serologic examination the organism was found to be a member of O group Y (O 47). It was agglutinated to titre by *S. bergen* O serum; in absorption tests all the O-agglutinins were removed from this serum. An O serum prepared with the new type (titre 1 : 10.000) agglutinated *S. bergen* to titre; in absorption tests there remained a residue of agglutinins for the homologous strains (1 : 32).

The H antigens of the new type were diphasic. Phase 1 was agglutinated strongly by *S. typhi* H serum; in absorption tests all H-agglutinins were removed. An H serum prepared with phase 1 of the new type (titre 1 : 10.000) agglutinated *S. typhi* to titre; in absorption tests there remained a residue of agglutinins for the homologous strain (1 : 250). Phase 2 was agglutinated by serum prepared with phase 2 of *S. springs*. An H serum prepared with

phase 2 of the new type (titre 1 : 640) agglutinated *S. springs* to titre; in absorption tests all H-agglutinins were removed.

The antigenic formula of the type was therefore 47 : d : z₃₉. As a *Salmonella* type with this formula is as yet not described, we propose the name *Salmonella quimbamba*.

We are indebted to Dr F. KAUFFMANN, International Salmonella Centre (Copenhagen), for the confirmation of our findings.

S u m m a r y.

A new *Salmonella* type, *Salmonella quimbamba*, isolated from fish-meal, was described. The antigenic formula is 47 : d : z₃₉.

(National Institute of Public Health, Utrecht, The Netherlands).

ESTABLISHMENT OF A DRIED STANDARD PERTUSSIS VACCINE

by

H. H. COHEN

(Received December 11, 1957).

The intracerebral mouse-protection test described by KENDRICK *et al.* (9) and by PITTMAN and LIEBERMAN (11) is the most widely used method for evaluating the potency of pertussis vaccine. Moreover, the results in the latest British fieldtrial¹⁾ seem to indicate that there is at least a reasonable correlation between potency in the mouse-protection test and the protective value of the vaccine in children. So it seems rational to follow the example set in the U.S.A. and to prepare international and national standard preparations with the aid of this test. In view of the extensive experience with the U.S.A. Standard (PITTMAN (12)), it seems indicated to evaluate the potency of new standards by comparing them with this U.S.A. vaccine. In fact this has been the policy of the Expert Committee W.H.O. with respect to the Proposed International Standard of Pertussis Vaccine. Moreover, the British Standard was included in the evaluation of this International Standard.

Mouse-protection tests being carried out on a large scale in our laboratory, the need was felt to prepare a vaccine which might be used as a laboratory standard. However, unfortunately, the larger part of our first lyophilized preparation (A), which was compared with U.S.A. Reference vaccine no. 5, was lost. This made it necessary to repeat the tests with a second preparation (B) and to assess its potency in comparison with preparation A, no American Reference vaccine no. 5 being available any more.

As the values obtained in the mouse-protection test vary greatly,

¹⁾ A report to the Whooping-cough Immunization Committee of the Medical Research Council. Brit. Med. J. ii, 454, 1956.

we doubted the accuracy of the result, this having been obtained only by indirect comparison with the U.S.A. Standard no. 4.

At this stage Dr M. PITTMAN, N.I.H., Bethesda Md., U.S.A. was so kind as to send us some ampoules of the U.S.A. Standard no 4, so that we could compare our preparation B directly with this standard in order to check the value found by us before. Dr PITTMAN also carried out some tests with our standard A and B herself. This form of co-operation made it possible to determine a definitive unit-value/ml for our preparation B. Some experiments were also carried out to compare our preparation B with the Proposed International Standard in our Institute and in the Statens Serum Institute in Denmark by Dr E. KRAG ANDERSEN.

In this paper the results of all these experiments are given. Moreover the problem is discussed of assigning to a standard a unit-value which is determined in a rather unreliable test.

MATERIAL AND METHODS.

Vaccines A and B. These were prepared from laboratory strain no. 512, which was known to yield a vaccine with a good protective value in the mouse-protection test.

The strain was cultured on the so called Glaxo-medium for 70 hours at 35°C. as described elsewhere (COHEN and LEPPINK (2)). Growth was harvested in saline. After centrifugation the supernatant was discarded and the bacteria were resuspended in 6 % dextran solution with 0.01 % merthiolate. Turbidity was adjusted to 50 opacity units by comparing it with the International pyrex opacity standard in an extincionimeter (VAN GENDEREN and SCHOLTENS (5)). The bacteria were killed by exposure to a temperature of 36°C. for 40 hours.

Ampoules were filled with exactly 1 ml and the contents were dried by lyophilization. After this the ampoules were melted off in vacuo. When needed the contents were resuspended in 5 ml saline with 0.01% merthiolate. All dilutions were made on a milliliter base.

Reference vaccine C. This was prepared from strain 134. The strain was cultured in the fluid Verwey medium (16) at 35°C. for 40 hours. The culture was centrifuged, the supernatant discarded and the sediment resuspended in dextran 6 % with 0.01 % merthiolate. The killing and lyophilization process was carried out in exactly the same manner as described for the vaccines A and B.

TABLE 1.

Doses of each vaccine expressed in ml given to each group of mice in the experiments given in table 2.

groups	Dutch experiments					U.S.A. experiments			Danish experiments	
	U.S.A. ¹⁾ R. 5	D.v.A.	D.v.B.	U.S.A. St. 4	Prop.Int. St.	D.v.C.	U.S.A. St. 4	D.v.A.	D.v.B.	Prop. Int. St.
1	0.030	0.250	0.125 or 0.250	0.250	0.125	0.125	0.200	0.200	0.050	0.01400
2	0.006	0.050	0.025 or 0.050	0.050	0.025	0.025	0.040	0.040	0.010	0.00280
3	0.0012	0.010	0.005 or 0.010	0.010	0.005	0.005	0.008	0.008	0.002	0.00056

¹⁾ U.S.A.R. 5 = Reference no. 5 (U.S.A.)

D.v.A. = Vaccine A (Dutch)

D.v.B. = Vaccine B (Dutch)

U.S.A. St. 4 = Standard no. 4 (U.S.A.)

Prop. Int. St. = Proposed International Standard (W.H.O.)

D. v. C. = Vaccine C (Dutch).

		3		7/20	9/19
	c	1		15/17	18/20
		2		14/20	14/20
		3		5/19	9/20
5 ³⁾	a	1	15/16	12/16	15/16
		2	3/16	7/16	8/16
		3	1/16	0/16	0/16
	b	1	10/16	13/16	15/16
		2	9/16	6/16	4/16
		3	0/16	1/16	1/16
	c	1	15/16	13/16	12/16
		2	5/16	6/16	6/16
		3	3/16	0/16	1/16
6 ⁴⁾	a	1		14/16	16/16
		2		8/16	10/16
		3		3/16	1/16
7	a	1		17/20	15/18
		2		8/19	8/20
		3		5/20	2/20
	b	1		18/20	18/20
		2		16/18	11/20
		3		4/19	4/17
	c	1		15/18	18/19
		2		11/20	10/19
		3		5/20	5/19
	d	1		12/20	15/19
		2		6/20	6/20
		3		2/19	2/20
					14/17
					8/18
					2/20
					17/19
					12/20
					8/19
					6/20
					5/20
					16/18
					15/17
					12/18
					11/19
					6/20
					8/20
					14/20
					14/19
					6/20
					7/20
					1/20

1) See table 1 for doses of vaccine in ml actually given.

2) U.S.A.R. 5 = Reference no. 5 (U.S.A.)

D.v.A. = Vaccine A (Dutch)

D.v.B. = Vaccine B (Dutch)

U.S.A.St. 4 = Standard no. 4 (U.S.A.)

Prop. Inst. St. = Proposed International Standard (W.H.O.)

D.v.C. = Vaccine C (Dutch)

3) Series 5 carried out by Dr M. PITTMAN, N.I.H., Bethesda Md., U.S.A.

4) Series 6 carried out by Dr E. KRAG ANDERSEN, Statens Serum Institute, Copenhagen, Denmark.

Mice. Swiss mice, bred in this Institute and weighing 10 - 14 gram, were used. The animals were segregated according to sex and divided by lot. Each group consisted of 20 or 40 animals, 10 (20) males and 10 (20) females.

Mouse-protection test. This test was carried out according to the N.I.H. specifications. Twenty (sometimes forty) mice in each of 3 groups were vaccinated intraperitoneally, each group with a five-fold increase of dose. The total amounts depended on the vaccine used. They are given in table 1 expressed in ml.

After a period of 12 days each mouse was given an intracerebral injection of 50.000 - 80.000 bacteria of KENDRICK's *H. pertussis* strain 18323, and then observed for another 14 days.

EXPERIMENTAL DESIGN.

As most of the ampoules of vaccine A were lost owing to a technical mistake, the experimental design could not be planned beforehand.

In the first set of 2 experiments (series 1¹) vaccine A was compared with U.S.A. Reference 5 (each experiment in duplo).

In a second set of 4 experiments (series 2) vaccine B was compared with vaccine A, no Reference vaccine no. 5 being available anymore (each experiment in duplo).

In a third set of 2 experiments (series 3) our vaccine B was compared with the U.S.A. master Standard no. 4 (each experiment in duplo).

In a fourth set of 3 experiments (series 4) our vaccine B was compared with the Proposed International Standard.

In series 5 the experimental results of Dr M. PITTMAN, N.I.H., Bethesda Md, U.S.A., comparing preparations A and B with U.S.A. Standard no. 4 are given.

In series 6 the experimental results of Dr E. KRAG ANDERSEN, Statens Serum Institute, Copenhagen, Denmark, comparing vaccine B with the Proposed International Standard are given.

In a seventh set of 4 experiments (series 7) our vaccine B was compared with our vaccine no. C.

¹) The results of the first 4 series were analysed statistically by Dr G. J. LEPPINK, Statistics Department T.N.O., The Hague, The Netherlands.

Results.

Results of the 7 series of experiments are shown in table 2 and are expressed in terms of the number of mice which survived per number injected in each group (for dosage of each vaccine see table 1). Probit analysis was used to find the best fitting straight line

TABLE 3.

ED₅₀ values of different vaccines in series 1 — 4 and 7, expressed in ml.

Series Exp.no.	U.S.A. R. 5	D.v.A.	D.v.B.	U.S.A. St. 4	Prop. Int.St.	D.v.C.	No.LD ₅₀ given in challenge dose
1 a	0.0838 0.1920	0.0870 0.0574					600
b	0.0228 0.0385	0.0249 0.0215					140
2 a		0.0726 0.0628	0.0138 0.0195				> 1000
b		0.1580 0.0998	0.0248 0.0419				450
c		0.3990 0.1810	0.0577 0.1270				580
d		0.0938 0.0734	0.0475 0.0312				700
3 a			0.0291 0.0236 0.0202	0.0597			220
b			0.0472 0.0982 0.0672	0.1340			840
4 a			0.0116		0.0125		80
b			0.0115		0.0078		150
c			0.0131		0.0070		< 100
7 a			0.0741 0.0428			0.0563 0.0472	> 1000
b			0.0174 0.0160			0.0158 0.0108	400
c			0.0182 0.0174			0.0139 0.0240	500
d			0.0244 0.0335			0.0315 0.0242	900

between the probit of the percentage of surviving mice and the logarithm of the dose. Two decimal points were carried in the probit values and in the weighting factors.

In table 3 the ED_{50} doses, obtained in series 1, 2, 3, 4, 7, calculated in ml are given for each vaccine. Moreover for each experiment an estimate is given of the no. of LD_{50} doses used as challenge (the LD_{50} value was determined by inoculating 3 control-groups of 12 mice with increasing numbers of germs and calculated roughly with the aid of the graphic probit method).

No correlation was found between ED_{50} and LD_{50} values. Further statistical analyses was carried out with the results of series 1 - 4.

With the aid of analysis of covariance was investigated, whether probit lines were parallel for each vaccine and for all experiments. The F quotients never exceeded the 5 % level of significance.

The 95 % confidence interval of the joint slope of the probit lines is $1.33 < b < 1.61$.

In table 4 the value of the slope for each vaccine is given.

TABLE 4.

Slope values and number of experiments from which they were calculated, for different vaccines.

Vaccine	Slope	Number of experiments from which the slope has been calculated
U.S.A. R 5	1.52	4
D. v. A.	1.40	12
D. v. B.	1.50	17
U.S.A. St. 4	1.82	2
Prop. Int. St.	1.16	3

The log ED_{50} values of the experiments in series 1 - 4 were computed by using the common slope $b = 1.47$. These values are given in table 5 for each vaccine and each experiment. The figures can be grouped according to 1) vaccine, 2) date of the experiment.

By means of the method of least squares the day effects were eliminated. The adjusted log ED_{50} values were calculated for each vaccine, and are given, expressed in ml, in table 6, together with their 95 % confidence limits.

As the Reference vaccine no. 5 contains 12.5 PU/ml and the master St.no. 4 1.25 PU/ml, values obtained with the reference vaccine were multiplied by 10. The adjusted means of ED_{50} values of the

TABLE 5.

Log ED₅₀ values of different vaccines calculated with the aid of the common slope $b=1.47$ for series 1—4 of the experiments.

series experi- ments vaccines	series 1		series 2				series 3		series 4	
	a	b	a	b	c	d	a	b	a	b
U.S.A. R 5 ¹⁾	— 1.07 — 0.81	— 1.66 — 1.43								
D. v. A.	— 1.09 — 1.25	— 1.62 — 1.69	— 1.15 — 1.22	— 0.70 — 1.00	— 0.71 — 0.89	— 1.00 — 1.10				
D. v. B.			— 1.61 — 1.53	— 1.58 — 1.23	— 1.35 — 1.26	— 1.32 — 1.47	— 1.55 — 1.63 — 1.71	— 1.27 — 1.20 — 1.15	— 1.97	— 1.90 — 1.87
U.S.A. St. 4							— 1.22	— 0.83		
Prop. Int. St.									— 1.91	— 2.00 — 2.02

¹⁾ to this value 1 was added (see text).

TABLE 6.
Adjusted means ED_{50} values with their 95% confidence limits.

Vaccine	Log ED_{50}	ED_{50} in ml	95% confidence interval of ED_{50}
U.S.A. R 5 ¹⁾	— 0.97	0.110	0.074 < ED_{50} < 0.15
D. v. A.	— 1.15	0.072	0.058 < ED_{50} < 0.089
D. v. B.	— 1.59	0.026	0.022 < ED_{50} < 0.031
U.S.A. St. 4	— 1.19	0.064	0.044 < ED_{50} < 0.093
Prop. Int. St.	— 1.65	0.023	0.016 < ED_{50} < 0.033

1) ED_{50} value multiplied with 10 (see text).

vaccines were compared with the aid of the method of SCHEFFÉ (14). The results are given in table 7. There is no significant difference between ED_{50} values/ml of Standard no. 4 and Reference no. 5.

TABLE 7.
Results of the significance test according to SCHEFFÉ for the adjusted treatment means.

D. v. A.	U.S.A. St. 4	D. v. B.	Prop. Int. St.	Vaccine
		⊕	⊕	U.S.A. R 5
		⊕	⊕	D.v.A.
		⊕	⊕	U.S.A. St. 4
				D. v. B.

⊕ = significant at $P = 0.05$.

DISCUSSION.

One of the most important requirements hitherto set for a biological standard preparation, is an exact determination of the unit value. This is certainly not the case with the standard preparations of Pertussis vaccine which are in current use internationally. The intracerebral mouse-protection test upon which the determination of the potency of these vaccines is based, gives varying results, as pointed out by many other authors. Our own observations confirm this. Originally we planned, using a large number of mice, to compare our vaccine A, with the U.S.A. Reference vaccine no. 5, which was then available in our laboratory. Accidentally, most of

the ampoules of vaccine A were lost, so the comparative experiments had to be discontinued. A new standard (B) was prepared, and the remaining ampoules of preparation A were used to compare preparation B with these. Thus vaccine B was a preparation of which the potency value was obtained only after three sets of comparative experiments (B with A, A with U.S.A. Ref. no. 5, U.S.A. Ref. no. 5 with U.S.A. Standard 4). At this stage it seemed interesting to us to check the result by comparing B directly with the U.S.A. Standard no. 4. This was done in our laboratory and by Dr M. PITTMAN, who compared our vaccines A and B directly with U.S.A. Standard no. 4.

Comparative investigations were also carried out between our vaccine B and the Proposed International Standard in our Institute and by Dr E. KRAG ANDERSEN.

Results obtained in Holland were divided into 4 series of experiments (table 1, 2, 3), which were analysed statistically. Moreover American and Danish results are given in these tables (series 5 and 6) and a comparative study of vaccine C and vaccine B (series 7).

In table 2 results are given as ED_{50} values of the experiments, each calculated with the aid of its own slope. In addition for each experiment the number of LD_{50} doses of the challenge dose is stated. In the different tests LD_{50} dose and ED_{50} dose of preparations A and B (with both vaccines a great number of experiments was performed) vary more than tenfold. There seems to be no correlation between LD_{50} and ED_{50} values, although results in continuous sets of experiments (see series 4) are indeed suggestive (see also COHEN (3)). It is realized that the conditions under which the test is carried out, can still be improved. Reduction of variation in LD_{50} dose will probably give more constant results.

In table 3 the ED_{50} value calculated for each separate experiment is given. Analysis of covariance showed that the hypothesis of equality of slope could be rejected neither for each vaccine separately nor for all vaccines together.

The common slope b of 1.47 (table 4) (sb 0.07) compares favourably with the value 1.28 found by us in a previous investigation (2) and is about the same as found in recent work of other authors (1.40 UNGAR (15); 1.29 KRAG ANDERSEN (10), which latter value must however be increased by 20 %, as she used the Wilson Worcester method for statistical analysis).

IRWIN and STANDFAST (7), analysing results of KENDRICK with the probit technique, found a pooled slope of 1.46 ± 0.09 . In their

own series of experiments slope values were much lower, namely 0.79, 0.73 and 0.93, respectively.

As the assumption of parallelism holds good, there is no objection to comparing the log ED_{50} values of the vaccines of the experiments of series 1 - 4 (table 5). By eliminating the day to day effects, an adjusted mean ED_{50} value for each vaccine could be computed (table 6).

In table 8 all results expressed in protective units/ml are pooled. Recently a value of 7 PU/ml was assumed by W.H.O. for the Proposed International Standard, as a weighted mean of the results of comparative assays of 9 laboratories. Compared with this standard and the U.S.A. Reference vaccine no. 5 the obtained values for the three vaccines tested seem to be definitively higher as after comparison with U.S.A. Standard no. 4. However the differences found between U.S.A. Reference no. 5 and U.S.A. Standard no. 4 were not statistically significant (table 6 and 7).

When fixing a definitive protective value for our own vaccine B, we chose the mean value of PITTMAN's result 4.3 and our own result (compared with U.S.A. Standard no. 4 = 3.1) = 3.7 PU/ml. The values found for vaccine A were in both laboratories nearly the same when compared with U.S.A. Standard 4 (1.1 and 1.2). Intentionally we disregarded the higher figure 5.3 found in the first 3 series of experiments for vaccine B, as the experimental conditions had been very complicated and the comparison not direct. This leads us again to the objections one can have against the assignment of a unitage to a standard preparation, which cannot be defined exactly by a biological test.

In U.S.A. this problem was solved by accepting vaccines with a relatively wide range of potency (8-36 PU/total immunizing dose; (minimum requirements¹⁾). As UNGAR (15) points out, the manufacturer must produce a vaccine with a specified minimum activity. Allowing for slight variations of manufacturing conditions and inaccuracy of biological assay, he should produce vaccines with potencies higher than the required minimum.

We realize that the value of 3.7 PU/ml is probably comparatively low. Compared with the Proposed International Standard (7 PU/ml) definitively higher values were calculated for vaccines A and B as

¹⁾ National Institutes of Health; minimum requirements: Pertussis Vaccine, 1st Revision, Bethesda (Md.) Oct. 31, 1952.

TABLE 8.

Results of comparative assays expressed in Protective Units/ml using different vaccines as standard.

Vaccines used as test-vaccines	Test lab.	No. of exp.	Prop. Int. St. (7 PU/ml) ¹⁾	No. of exp.	U.S.A. St. no. 4 (1.25 PU/ml)	No. of exp.	U.S.A. R. no. 5 (12.5 PU/ml)	No. of exp.	D.v. A.	No. of exp.	D.v.B.
D.v.B.	N.I.H. Holland	3	$\frac{230}{260} \times 7 = 6.2$	2	$\frac{64}{26} \times 1.25 = 3.1$		$\frac{11}{26} \times 12.5 = 5.3$	4	$\frac{720}{260}$		
	N.I.H. U.S.A.			3	4.3						
	St. Serum Inst. Den-mark	1	$\frac{21}{25} \times 7 = 6.7$								
D.v.A.	N.I.H. Holland	2)	$\frac{23}{72} \times 7 = 2.2$	2)	$\frac{64}{72} \times 1.25 = 1.1$	2	$\frac{11}{72} \times 12.5 = 1.9$			4	$\frac{260}{720}$
	N.I.H. U.S.A.			3	1.2						
D.v.C.	N.I.H. Holland									4	mean value of 4 × 2 experiments 4.1 (table 9)

¹⁾ ED₅₀ values of vaccines (table 6) × 10⁴.²⁾ Not directly compared (see table 6).

well in our laboratory as in the Statens Serum Institute (table 8).

However, in our laboratory, using a value of 3.7/ml, we nearly always succeed in producing vaccines with a protective value of between 8-36 PU/total immunizing dose (60.000 millions of germs). As vaccines with a protective value below 12 (JAFJE (8), PITTMAN (13)), seem to give less favourable immunizing results in children, in contrast with vaccines with values higher than 12 units (FELTON *et al.* (4)), there is no reason for us to run the risk of lowering our manufacturing standard by adopting a higher potency value for our vaccine.

ARMITAGE and PERRY (1) followed an alternative and attractive method for the assesment of the British Standard vaccine. They base their requirements on the correlation existing between the intracerebral mouse-protection test and the reduction of the home exposure attack rate, found in the latest British fieldtrial. These authors propose to accept vaccines which reduce this home exposure attack rate from 80-90 % to 4-30 % and accordingly have set the requirement for estimated potency somewhere between 1.3 and 6.4 times the dried British Standard. In the comparative W.H.O. investigation, in which nine laboratories participated the weighted average of the British Standard vaccine was found to be about 1 unit/ml. In this way American and British minimum requirements can be compared.

From the foregoing it will be clear that potency standards for pertussis vaccines occupy an exceptional position among biological standard preparations. However, it is generally realized that the standard cannot be dispensed with in maintaining and controlling the quality of pertussis vaccine production. Producers must realize, that the standard cannot be used to claim a definite number of protective units for their products. But to indicate the quality of their products they must be allowed to label them as: „containing between 8-36 PU/total immunizing dose”, or when one prefers to place only one value „containing 12 PU/total immunizing dose”.

In the last set of 4 experiments (table 2, 3, series 7) we compared a lyophilized reference vaccine C with our standard vaccine B. No common slope was calculated, and ED₅₀ values obtained in duplicate in each experiment were calculated in units/ml (assuming a value of 3.7/ml for vaccine B). These values are given in table 9. From these results it is clear that under favourable conditions reasonably accu-

rate results can be obtained in the mouse-protection test, the extreme values differing $\pm 100\%$.

TABLE 9.

Protective values expressed as units/ml of vaccine C as compared with vaccine B (series 7 of experiments).

Series 7 Exp.	Protective values in duplicate	
a	3.4	4.4
b	4.0	5.9
c	2.7	4.7
d	3.7	4.4
Geometric mean 4.1		

In this discussion we expressed all definitive results in protective value per ml. This is in accordance with the definition of potency by IRWIN (6): „The unit is defined as the specific biological activity of a given amount of the standard”.

It seems better to avoid expression of potency in turbidity units and to refrain from the actual number of bacteria to a ml. The definition of a unit of turbidity depends on optical measurement of a suspension of Pyrexglass particles in spectrophotometers of different origin. So the possibility of differences between these turbidity measurements cannot be excluded. Moreover *B. pertussis* vaccines show a tendency to lyse after several months of cold storage, whereas the potency depending on the total amount of antigen present, remains constant.

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Summary.

The potency of an own Dutch standard pertussis vaccine B was determined by indirect comparison in three stages (B with A;

A with U.S.A. Reference no. 5; U.S.A. Reference no. 5 with U.S.A. Standard no. 4) with the U.S.A. Standard no. 4 in the intracerebral mouse-protection test. The values found were checked by direct comparison with U.S.A. Reference no. 4 in Holland and U.S.A.

In this way a definitive value of 3.7 units/ml was given to this standard. The reasons are discussed for giving a unit value to a standard, although this unit value cannot be determined accurately in a biological test.

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THE USE OF CHLORHEXIDINE

by

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Disinfectants play a most important role in hospitals in the combating of infectious diseases. The number of disinfectants with a wide range of application is very limited. From data received, chlorhexidine (or 1:6-di-4-chlorphenyldiguanidohexane) promised an extensive field of application and it was therefore decided to submit it to bacteriological clinical tests.

The first publication on chlorhexidine is from DAVIES, FRANCIS, MARTIN, ROSE and SWAIN (1954). Chlorhexidine is available as the dihydrochloride and the diacetate. The diacetate is soluble to the extent of 1.9% in distilled water at 20°C.; the dihydrochloride to only 0.06% at this temperature. Antibacterial activity is the same for both salts. The bacteriostatic concentrations are given in Table 1, which is taken from the article of DAVIES *et al.*

We studied the bacteriostatic concentrations of chlorhexidine required for certain organisms, and our results (Table 2) correspond with those obtained by DAVIES and his colleagues.

These tests were carried out using a 1% glucose broth medium at pH 7.6. This was inoculated with 0.1 ml of a 6-hour culture diluted 10 times with 1% glucose broth. For the tests with *Streptococcus pyogenes* and *Corynebacterium diphtheriae* a 10% sheep serum broth was employed and the bacterial culture was 20 hours old.

According to I.C.I. reports, chlorhexidine kills 99.99% bacteria in 10 minutes at the concentrations indicated in Table 3.

TABLE 1.

Microorganism	Minimum concentration inhibiting growth after 24 hours at 37° C.
<i>Bacillus subtilis</i> (1)	1 : 1.000.000
<i>Streptococcus lactis</i> (1)	1 : 2.000.000
<i>Streptococcus pyogenes</i> (4)	1 : 500.000 — 1 : 1.000.000
<i>Corynebacterium diphtheriae</i> (1)	1 : 500.000
<i>Diplococcus pneumoniae</i> (1)	1 : 500.000 — 1 : 1.000.000
<i>Micrococcus pyogenes</i> (20)	1 : 500.000 — 1 : 2.000.000
<i>Proteus vulgaris</i> (1)	1 : 200.000
<i>Salmonella pullorum</i> (1)	1 : 300.000
<i>Vibrio cholerae</i> (1)	1 : 200.000
<i>Streptococcus faecalis</i> (1)	1 : 200.000
<i>Salmonella Dublin</i> (1)	1 : 100.000
<i>Salmonella typhi-murium</i> (1)	1 : 100.000
<i>Aerobacter aerogenes</i> (1)	1 : 100.000
<i>Bacterium coli</i> (1)	1 : 100.000
<i>Pseudomonas pyocyanea</i> (10)	1 : 50.000 — 1 : 100.000

The numbers in parenthesis denote the number of strains tested.

TABLE 2.

Microorganism	Minimum bacteriostatic concentration of chlorhexidine
<i>Streptococcus pyogenes</i> (Lancefield group A. type 3)	1 : 2.000.000
<i>Micrococcus pyogenes</i>	1 : 1.000.000
<i>Corynebacterium diphtheriae</i> (gravis)	1 : 1.000.000
<i>Diplococcus pneumoniae</i>	1 : 1.000.000
<i>E. coli</i>	1 : 1.000.000
<i>Ps. pyocyanea</i>	1 : 300.000

TABLE 3.

Microorganism	Concentration of chlorhexidine
<i>Micrococcus pyogenes</i>	1 : 40.000
<i>E. coli</i>	1 : 50.000
<i>Ps. pyocyanea</i>	1 : 17.500
<i>S. typhi</i>	1 : 125.000

The inoculum contained approximately 100 million organisms. Surviving organisms were further investigated by DAVIES and his colleagues and they failed to isolate strains resistant to chlorhexidine even after subculture.

It can be concluded from the foregoing that chlorhexidine is powerfully bacteriostatic and bactericidal, at least for non-sporing bacteria.

The activity of chlorhexidine is diminished in the presence of pus. It is inactivated by anionogenic substances such as lecithin in large concentration, soap and sodium carboxymethylcellulose, whereas in vitro, it is not inactivated by the presence of sulphonamides, penicillin, aureomycin and chloramphenicol. The Rideal-Walker coefficient is 750 and the Chick-Martin coefficient is 19. The toxicity for mice being 5 mg/kilo body weight, men used 2 g oral, without symptoms, during 1 week.

The maximum oral dose for man is 100 mg/kilo body weight. Chronic toxicity tests in rats gave similarly satisfactory results; and these animals given drinking water containing chlorhexidine at a concentration of 1 : 2,000 showed no abnormality up to 3 generations. The satisfactory bactericidal and bacteriostatic properties of chlorhexidine encouraged us to try it in urological practice. In 1956, BEEUWKES and DE VRIES (1956) reported favourably on its use during urological procedures. They employed a 1 : 10,000 solution of chlorhexidine dihydrochloride in tap water as an irrigating fluid; a 1 : 5,000 solution for bladder irrigation; 1 : 1,000 for disinfection of the external genitalia; and 1 : 1,000 solution of the diacetate compound for cotton wool swabs. Between cystoscopies the tap of the irrigator tube and the switch for the light were dipped in a 1% solution of chlorhexidine in 70% alcohol.

Finally, the operating theatre staff rubbed their hands several times a day with a preparation of 1% chlorhexidine in a polyethyleneglycol base ('Sterilon'). Tests with this cream showed that with an 8 mm hole in broth agar sown with *Micrococcus pyogenes*, an inhibitory zone 21 mm in diameter was obtained; with *Ps. pyocyanea* the zone was 18 mm and with *E. coli* 19 mm in diameter.

Following the introduction of this technique there was no more urinary infection due to *Ps. pyocyanea*, and in 8% of 50 patients only could *Staphylococcus albus* be cultured from the urine. Before these measures took effect, the corresponding figures were 56% for *Ps. pyocyanea*, 10% for the *Proteus* group and 4% for *Staphylococcus albus*. 30% of urines were sterile.

Further for disinfection, chlorhexidine may be mixed with glycine (100 g glycine with 1 g chlorhexidine in 10 litres of tap water) and

then used as an irrigating fluid in trans-urethral resection. According to DE VRIES this much improves the post-operative outlook. We therefore conclude that chlorhexidine is of great therapeutic value in urological procedures.

It might be anticipated that this substance would act as an efficient surface disinfectant. According to MURRAY and CALMAN (1955) a 1% chlorhexidine cream diminishes the number of bacteria on the skin, and they recommend its use in laboratories and hospitals in order to combat cross infection.

MEDICAL EXPERIMENTS.

Pediatrics. To investigate this reported activity, we conducted the following investigation. In the children's department (Head Dr O. A. DRIESSEN), alternate newly born infants, after their first bath, were rubbed with Cetavlon cream (I.C.I.). Of the 38 not so treated, 4 had pemphigus neonatorum, while of the 48 treated with the cream, 12 developed pustules. We then treated alternate infants of a series of 78 with 0.5% G. 11 (Hexochlorophene) cream (Brocades Stheeman). Of the 39 not treated 37 had pemphigus neonatorum, while in the same time only 7 of the treated infants developed pustules.

Some months later, we instituted this routine treatment using chlorhexidine cream. The same favourable results were obtained, i.e. the almost complete disappearance of pemphigus neonatorum and the absence of other staphylococcal infections. This satisfactory situation has been maintained for more than $1\frac{1}{2}$ year, and we conclude that chlorhexidine cream is very useful for prophylaxis against skin infections in infants. We prefer chlorhexidine to G. 11 because the latter is active chiefly against gram positive organisms.

Burns. Chlorhexidine will probably prove of value in the treatment of burns. The incidence of sensitivity towards it ought to be small, while the development of bacterial drug resistance may prove to be insignificant. LOWBURY (1955) investigated the action of chlorhexidine in burns. In a series of 33 cases treated with penicillin cream, *Staphylococcus aureus* was recovered from no less than 85%, whereas in a series 29 treated with chlorhexidine cream (1 mg/g) this organism was grown from the wound exudate in only 21%.

LOWBURY also points out that the classical methods of preventing cross infection, including isolation in cubicles and the aseptic technique, are inadequate to prevent the emergence of resistant staphylococci among hospital patients. He concludes his investigations with the following observations: "Although our attention today is drawn to combined chemotherapy as the most hopeful weapon against the emergence of resistant forms, it is too early to assume that no substance will be discovered which is as safe as penicillin and as permanent in its effects against *Staph. aureus* as phenol, and perhaps hitbitane".

In order further to assess the value of chlorhexidine as a surface disinfectant, we asked the Heads of the various departments to try this substance instead of tincture of iodine.

Obstetrics and gynaecology. In the Department of Obstetrics and Gynaecology, with Dr TH. J. VAN SANTE in charge, chlorhexidine is used in a number of ways as follows. The skin is disinfected with 0.5% chlorhexidine in 50% alcohol, without previous washing. In abdominal operations, after the external genitals have been washed with G. 11 soap, irrigation is performed once with a 1 : 5,000 solution of chlorhexidine in distilled water. In vaginal operations, douching is performed once with chlorhexidine 0.5% in 50% alcohol. Swabs are soaked in a 1 : 1,000 solution. For a bladder wash-out a 1 : 10,000 aqueous solution is used, since a 1 : 5,000 solution occasionally causes bladder spasm. Chlorhexidine was used for more than a year, in a total of 100 vaginal and 100 abdominal operations, and during that time no single case of wound infection was encountered.

Urology. In the department of urology, under Dr H. R. DE VRIES, chlorhexidine was tried as a surface disinfectant in place of tincture of iodine and mercurochrome. The operation area is disinfected with 0.5% chlorhexidine in 50% alcohol, and before closing a contaminated wound for example, it is washed out with an aqueous solution of 1 : 1,000. The results obtained here have also been striking, as evidenced by a considerable decrease in the incidence of wound infections. The use of chlorhexidine is especially recommended in operations such as vasectomy or orchidectomy and others requiring a trans-scrotal approach. Before returning the scrotal contents the tissues are bathed with 0.5% chlorhexidine in 70% alcohol.

Surgery. In the department of Surgery, directed by Dr F. J. A. BUYTENDYK, the skin is disinfected by five minutes application of 0.5% chlorhexidine in 50% alcohol, without previously washing with soap:

In an operation for acute appendicitis, after closing the peritoneum, the wound is first cleaned with hydrogen peroxide and then bathed with a 1% aqueous solution of chlorhexidine. Experience with the use of this substance in burns is still not extensive enough to warrant a final opinion. The preparation used in this indication is a soft, almost fluid cream (polyethylene glycol) containing 1% chlorhexidine.

MICROBIOLOGICAL EXPERIMENTS.

In considering the results described above, the question immediately arises, how can any conclusion be drawn when no control cases were observed? In that situation judgement is necessarily founded on clinical impressions. The experience gained by the departments concerned was so favourable that the use of both tincture of iodine and mercurochrome has been abandoned.

The powerful surface disinfecting action of chlorhexidine is further demonstrated by the experiments of Dr DE ROM in Ghent. He investigated the number of living bacteria present on the shaved skin of guinea pigs after two minutes contact with a solution of 1% chlorhexidine in 50% alcohol. Drops of cultures of *Micrococcus pyogenes*, *E. coli* and *Ps. pyocyanea* were each placed on the skin of 6 guinea pigs. A series of 6 untreated animals served as controls. Cultures from the treated series did not show any growth, in contrast with the confluent growth obtained from the control animals.

Micrococcus pyogenes. In this connection the following in vitro experiments are of interest. 0.05 ml of a 6-hour culture of *Micrococcus pyogenes* was put on each of the rubber caps of 6 bottles, each of 60 ml capacity, and each containing about 40 ml of broth. When the culture had dried, 0.05 ml of a) 3% tincture of iodine in 70% alcohol, b) 5% tincture of iodine in 70% alcohol, and c) 0.5% chlorhexidine in 50% alcohol, were placed on 3 separate bottle caps. The other 3 bottles, with bacterial culture only on the rubber caps, served as controls. After exactly 60 seconds, each of the rubber caps was punctured with a sterile hypodermic needle No. 2, so that the broth within the bottles came in contact with the needles. The

bottles were kept at 37° C. for 24 hours. In the control bottles only bacterial growth had occurred. The experiment was repeated, using 1% chlorhexidine and a 24 hours old bacterial culture. The bottles treated with chlorhexidine remained sterile, while *Micrococcus pyogenes* grew in the broth of the control bottles. The method described is a slight modification of that used by Ruys (1954).

Fungi. In order to get an impression of the fungistatic and fungicidal activity we made the following experiments. The diacetate compound was diluted with Sabouraud's dextrose culture medium. This was inoculated with 0.1 ml of a mould suspension prepared thus: 10 ml tryptose-phosphate broth were added to a 14-day old culture on an agar slope kept at room temperature. The mould culture was suspended with a grafting needle, and after vigorous shaking, filtered through sterile hydrophyl gauze. The results were recorded after 14-days at room temperature.

TABLE 4.

Organism	Growth	No growth
<i>Trichophyton asteroides</i> Sabouraud	1 : 10.000	1 : 1.000
<i>Trichophyton interdigitale</i> Priestly	1 : 10.000	1 : 1.000
<i>Trichophyton Schönleinii</i> (Lebert)		
Langeron et Milochevitch	1 : 100.000	1 : 10.000
<i>Trichophyton tonsurans</i> Mahusten	1 : 100.000	1 : 10.000
<i>Trichophyton rubrum</i> (Cast.) Sab.	1 : 10.000	1 : 1.000
<i>Trichophyton violaceum</i> Bodin	1 : 100.000	1 : 1.000
<i>Trichophyton verrucosum</i> Bodin		
var. <i>discoïdes</i> (Sab.) Georg.	1 : 10.000	1 : 1.000
<i>Trichophyton ferrugineum</i> (Ota) Talice	1 : 10.000	1 : 1.000
<i>Aspergillus fumigatus</i> (Fres.) Berkhout	1 : 10.000	1 : 1.000
<i>Aspergillus niger</i> v. Tieghem	1 : 10.000	1 : 1.000
<i>Microsporium felineum</i> Fox et Blaxall	1 : 100.000	1 : 10.000
<i>Microsporium audouini</i> Gruby	1 : 10.000	1 : 1.000
<i>Epidermophyton flocculosum</i> (Harz)		
Langeron et Milochevitch	1 : 100.000	1 : 10.000
<i>Candida albicans</i> (Robin) Berkhout	1 : 10.000	1 : 1.000

All the organisms used in this experiment were obtained from the Centraal Bureau voor Schimmelcultures in Baarn.

It can be observed from this table that for four organisms growth was inhibited at a dilution of 1 : 10.000 and in 10 there was no growth at a 1 : 1.000 dilution.

We examined the fungicidal activity of this substance against *Trichophyton interdigitale* and *Candida albicans*. The mould culture was 14 days old and the suspension was prepared in the manner described above. To 1.0 ml of the suspension we added 1.0 ml of a) 5% chlorhexidine in 50% alcohol, b) 1% chlorhexidine in 50% alcohol, and c) 50% alcohol, to act as a control.

After 60 seconds and after 10 minutes, we mixed 0.5 ml of this mixture with 2.5 ml of egg yolk broth, prepared by suspending 1 egg yolk in 100 ml of tryptose-phosphate broth. 0.5 ml of this mixture was spread with a spatula on Sabouraud agar in a Petri dish of 12 cm diameter. The cultures were observed after 10 days, that is as soon as the control plate showed growth. The control plate was entirely overgrown with moulds. 1% chlorhexidine in alcohol, after 60 seconds contact, did not affect growth, but after 10 minutes contact there were only 33 colonies. 5% chlorhexidine inhibited growth after both 60 seconds or 10 minutes contact.

The inoculum of *Candida albicans* consisted of approximately 2,000,000 vegetative cells and spores of a 14-day old culture. The control plates were once more entirely overgrown, whereas after contact with 1% chlorhexidine for either 60 seconds or 10 minutes there was no growth. The same result obtained after 60 seconds exposure to 1% chlorhexidine when 30% egg yolk was included in the Sabouraud culture medium. There was no noticeable difference if the inoculation was made onto Sabouraud plates without added egg yolk. The same result was obtained if the yeast suspension, after 60 seconds contact with 5% chlorhexidine, was washed once with 30% egg yolk suspension, and then inoculated onto Sabouraud plates both with and without added egg yolk. DAVIES and his colleagues demonstrated that tubercle bacteria are 1,000 times less sensitive when grown on Lowenstein's culture medium of which egg yolk is an important constituent, than when grown in Dubos' liquid culture medium. Activity was found to be decreased about 100 times in the case of *Micrococcus pyogenes*, and 10 times in the case of *Candida albicans*.

The fungistatic activity of chlorhexidine is further demonstrated by diffusion tests. For this purpose we investigated the action of chlorhexidine-cream and undecylene ointment on *Trichophyton mentagrophytes*. Using an 8 mm hole in broth agar, the inhibitory zones were both 18 mm in diameter (see Fig. 1).

It is striking that the inhibitory zone produced by chlorhexidine was not overgrown by *Trichophyton mentagrophytes*, in contrast with that of the undecylene ointment (Brocades Stheeman). We considered the possibility that chlorhexidine might be of value in the treatment of fungus diseases of the skin, and a clinical investigation was therefore carried out by Prof. L. VAN DER MEIREN and

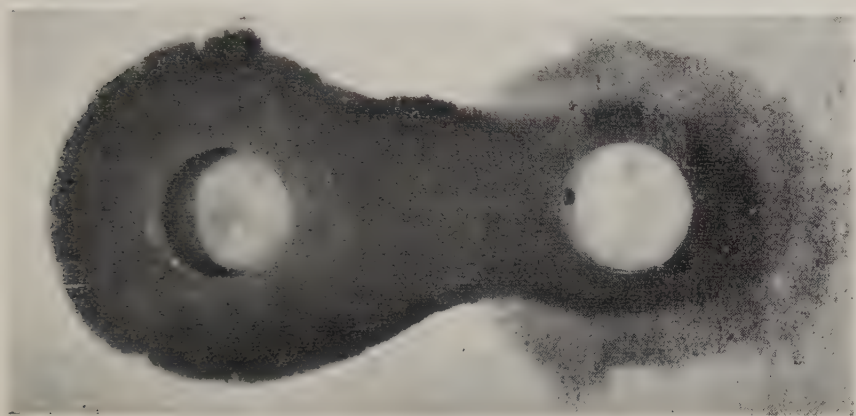


Fig. 1.

Chlorhexidine-cream.

Undecylene ointment.

Dr G. ACHTEN of the Université Libre, Brussels. The results that they provisionally obtained were as follows: In 9 cases of fungus infection, 5 were cured, 2 improved and 1 remained unchanged; 1 patient was intolerant of the treatment. In 12 cases where *Candida albicans* was assumed to be the cause, 5 were cured, 5 improved and 2 remained unchanged. Treatment consisted of applying 5% chlorhexidine in 50% alcohol in the morning and 5% chlorhexidine in polyethylene glycol in the evening. The investigators considered the results obtained to be favourable, especially in view of the fact that chronic cases were included in the series.

β -haemolytic streptococci. Finally, we considered whether chlorhexidine might be useful in the prophylaxis of mouth infections caused by β -haemolytic streptococci. In this connection it is essential to determine whether a lozenge, containing a disinfectant, can produce adequate disinfection in the mouth. We therefore carried out the following tests with a number of oral disinfectant tablets,

using a technique that we developed. The intention was to investigate the concentration of chlorhexidine in saliva that would be active against β -haemolytic streptococci.

Experiment 1.

Two ml of saliva were mixed with 50 γ chlorhexidine. To this was added 0.1 ml of a 24-hour culture of streptococci (S.84 type 3, Lancefield group A) grown in 10% sheep serum broth. After 3 minutes, this was inoculated onto a 5% sheep's blood agar plate and after incubating for 24 hours at 37°C. the number of colonies was estimated. In this experiment with chlorhexidine 20 colonies had grown, whereas in a similar experiment substituting Cetavlon (I.C.I.) for chlorhexidine, innumerable colonies of β -haemolytic streptococci were visible.

Conclusion. Chlorhexidine is highly antibacterial for β -haemolytic streptococci in saliva, in contrast with Cetavlon, which consists of a trimethylammoniumbromide nucleus with various alkyl groups attached, and is one of the quaternary ammonium bases.

Experiment 2.

To 3 ml of 10% serum broth is added 25 γ chlorhexidine per ml and then 0.1 ml of bacterial culture. After 3 minutes, this is inoculated onto a blood agar plate. The control plate showed innumerable colonies, whereas that inoculated from the tube containing chlorhexidine showed no growth. A tube containing chlorhexidine and bacterial culture receives, 3 minutes later, 0.3 ml; 0.2 ml, and 0.1 ml, respectively of egg yolk. After 20 minutes, 0.1 ml of the tube contents is inoculated onto a blood agar plate. The control plates always showed innumerable colonies, whereas of the plates inoculated from the tube containing 0.3 ml egg yolk, approximately 50% grew β -haemolytic streptococci. Inoculation from the tubes containing 0.2 ml or 0.1 ml egg yolk showed no growth. We repeated this investigation with *Micrococcus pyogenes* (isolated from a boil) and with *Corynebacterium diphtheriae* (gravis) using 30 γ chlorhexidine per ml. The inoculum was 0.1 ml of a 24-hour culture in 1% glucose broth and 10% sheep serum broth respectively. The inoculation was performed after 3 minutes contact, both with and without the addition of 10% egg yolk. Contact with chlorhexidine for 3 minutes inhibited the growth of *Corynebacterium diphtheriae* and β -hae-

molytic streptococci, and the egg yolk had no perceptible influence. After 3 minutes contact with chlorhexidine, *Micrococcus pyogenes* gave approximately 40% growth in the absence of egg yolk, but about 70% growth when egg yolk was included.

Conclusion: Chlorhexidine, in a strength of 25 γ per ml is obviously bacteriostatic against organisms in saliva, and at a strength of 30 γ per ml is markedly bactericidal against β -haemolytic streptococci. The inoculum of β -haemolytic streptococci consisted of about 60,000,000 bacteria, so that there were approximately 20,000,000 per ml. The inoculum of *Micrococcus pyogenes* and *Corynebacterium diphtheriae* was not numerically estimated.

Experiment 3.

Chlorhexidine concentration in saliva after sucking tablets containing 2 mg chlorhexidine per tablet. Immediately after sucking 1 tablet, 1.0 ml of saliva was diluted with 10% serum broth to a concentration of 1 : 2,560. To each 1.0 ml of this mixture is added 0.05 ml of bacterial culture and 0.1 ml of 5% sheep's blood. A second series of tubes is set up containing the following concentrations of chlorhexidine: 5 γ /ml, 2.5 γ /ml, 1.25 γ /ml, 0.625 γ /ml, 0.312 γ /ml and 0.15 γ /ml. After 24 hours, a 1 : 160 dilution of saliva showed no growth of β -haemolytic streptococci following inoculation on to a blood agar plate, and a concentration of 2.5 γ /ml chlorhexidine similarly showed no growth. If the subject swallows as little saliva as possible while sucking the tablet, it is possible to attain a concentration of approximately 400 γ /ml chlorhexidine of saliva. Obviously, this figure depends very much on the manner in which the tablet is sucked and whether or not the saliva is swallowed.

Experiment 4.

The antibacterial activity of a number of lozenges against β -haemolytic streptococci was then investigated. Immediately after the various tablets had been sucked, 0.1 ml of saliva and 0.05 ml of a 24-hour culture of β -haemolytic streptococci (S 84 Lancefield group A) are mixed. Three minutes later, 0.05 ml of the mixture is withdrawn and spread with a spatula on a 5% sheep's blood agar plate and incubated for 24 hours at 37° C. A control experiment was set up for each tablet, and the control plates all showed the

growth of innumerable colonies. The results following the sucking of the various tablets were as follows:

1 mg nitrofurazone (Angiletten)	innumerable colonies
2 mg dichloroxychinaldine (Siogen)	innumerable colonies
1 mg tyrothricin (Tyrosolven lozenge)	\pm 50% growth
3 mg 3-6 diamino-10-methylacridine-chloride (Pantonsiletten)	\pm 30% growth
1 mg penicillin (penicillin throat tablet)	5 colonies
2 mg chlorhexidine (Rotersept)	10 colonies
organic iodine compound (Agré Gola)	innumerable colonies
0.25 mg Dequadin	innumerable colonies

From these results it appears that the penicillin lozenge and the chlorhexidine tablet are most active. Angiletten, Siogen, Agré Gola and Dequadin do not exhibit any antibacterial action in saliva when they are sucked, if care is taken to swallow as little saliva as possible.

In assessing the results of these experiments, due allowance must be made for the subjective element involved. These qualitative results were then confirmed by a quantitative investigation. Of the 8 tablets examined, the penicillin lozenge, the chlorhexidine tablet and Pantonsiletten exerted an average bacteriostatic activity in 10% sheep serum broth of 1 : 140, 1 : 33 and 1 : 46 respectively; the remaining tablets showed no activity at an initial dilution of 1 : 10.

The determinations were always carried out on 3 subjects, and the following were the inhibiting concentrations of the most active tablets:

Penicillin lozenge	1 : 320	1 : 80	1 : 20
Chlorhexidine tablet	1 : 40	1 : 40	1 : 20
Pantonsiletten	1 : 80	1 : 40	1 : 20

In these tests the saliva was swallowed normally while the tablets were being sucked.

Conclusion: The penicillin and the chlorhexidine tablets, after 3 minutes contact show a satisfactory antibacterial activity in saliva towards β -haemolytic streptococci. From the dilution test results, however, Pantonsiletten are also clearly bacteriostatic. The remaining tablets were quite inactive.

Chlorhexidine is preferable to penicillin, because patients may develop a sensitivity to penicillin, and there is the possibility that oral organisms may become resistant to this antibiotic. The 3-6

diamino-10-methylacridinechloride obviously had a bacteriostatic action, but was clearly less effective in the qualitative saliva test, in which, after 3 minutes contact, the material was inoculated on to a blood agar plate. We must consider whether the results of this test are a sound argument for preferring chlorhexidine, remembering that with 3-6 diamino-10-methylacridinechloride substantial bacteriostatic concentrations can also be achieved in the saliva. In our view, a substance lethal to bacteria in the mouth must act quickly and effectively. This test, in which inoculation is arbitrarily done after 3 minutes contact, supports this opinion. Further, it is important that chlorhexidine has no detrimental effect on the flora of the mouth, and that oral bacteria, as far as we know, do not become resistant towards it. The remaining tablets all gave unsatisfactory results in vitro by the methods described above. It is obvious that clinical trials must be carried out in human beings to confirm the action of chlorhexidine on β -haemolytic streptococci.

Field trial. A double blind clinical trial of this nature was conducted with the kind cooperation of Colonel BEUNDERS, M. D. and the Garrison Medical Officer BASTIANEN.

Swabs were taken from the throats, particularly from the tonsils of 619 recruits and were examined within 2 hours for the presence of β -haemolytic streptococci. They were inoculated on to 5% sheep's blood agar plates and grown both aerobically and anaerobically. Haemolytic streptococci grew in 89, that is in 17% of subjects.

Thirty four subjects received 2 tablets of Rotersept 5 times daily for a week, while 39 control subjects received placebo tablets in similar dosage. Throat swabs were taken on the 8th day. It was found that in the group receiving placebo, 23 subjects showed haemolytic streptococci in their throats, whereas of those who were taking the Rotersept tablets, only 9 were positive for the organism. This was a statistically significant result.

This investigation suggests that further enquiry into this problem is desirable. It may well be that regular use of chlorhexidine tablets is of prophylactic value against β -haemolytic streptococci.

S u m m a r y.

An investigation was carried out on the therapeutic value of chlorhexidine (I.C.I.). We are of the opinion that this substance,

which has very few toxic side-effects, may prove to be of great importance as a general disinfectant in surgical practice, of prophylactic value in paediatrics, in urological practice, in the treatment of burns, and generally to prevent cross infection. Further, it was established that chlorhexidine possesses fungistatic and fungicidal action.

A preliminary investigation with chlorhexidine showed a favourable therapeutic response in skin diseases due to fungi and *Candida albicans*.

In vitro tests suggest that chlorhexidine may be of value in the mouth to combat β -haemolytic streptococci, without influencing the oral flora.

Acknowledgements.

Chlorhexidine is manufactured by Imperial Chemical Industries, and marketed under the name of "Hibitane".

We are grateful to I.C.I. for information concerning this drug and the supplies used in this investigation.

Supplies of 1% chlorhexidine in a polyethylene glycol base, marketed under the name "Sterilon" were kindly made available to us by the Roter Company to whom we offer our thanks.

We thank Dr PAUL DE ROM for allowing us to quote the results of his investigations which he very kindly placed at our disposal.

We also thank Professor L. VAN DER MEIREN and Dr G. ACHTEN for permission to quote the results of their investigations with chlorhexidine.

Rotersept throat tablets used in this investigation were made available to us by the courtesy of the Roter Company, Hilversum, and we gratefully acknowledge their supplies.

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THE VITAMIN REQUIREMENTS OF LACTIC ACID BACTERIA FROM CIDERS

by

J. G. CARR

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INTRODUCTION.

During a survey of the lactic acid bacteria of ciders and apple juices certain strains were tested for their vitamin requirements. LÜTHI (1957) has shown that *Bacterium gracile*, a lactic coccus which commonly occurs in Swiss wines and ciders, requires pantothenic acid, nicotinic acid, riboflavin and folic acid for growth. Although similar information has been recorded about brewery lactic acid bacteria by RAINBOW (1952); RUSSELL, BHANDARI and WALKER (1954); MOORE and RAINBOW (1955) the work of LÜTHI represents the only data concerning organisms occurring in cider. It was, therefore, necessary to extend the present information about the nutritional requirements of lactic acid bacteria occurring in apple juice and cider.

METHODS.

The test medium was prepared in two parts, namely a basal medium and a vitamin mixture. The basal medium contained the following per 1000 ml: acid hydrolysed casein (Allen & Hanbury's), 10 g; glucose, 10 g; L-arabinose, 10 g; adenine, guanine, uracil and xanthine, each 0.012 g; tryptophan, cysteine and γ aminobutyric acid, each 0.667 g; Tween 80, 0.100 g; KH_2PO_4 , 0.550 g; KCl 0.425 g; CaCl_2 and MgSO_4 , each 0.125 g; FeCl_3 and MnSO_4 , each 0.0025 g.

Mixtures were prepared from vitamin stock solutions. One of these was complete whilst each of the others was deficient in a single vitamin. Organisms were tested for their requirements of the following vitamins: thiamine; B_6 (pyridoxine, pyridoxamine, pyridoxal); calcium pantothenate; riboflavin; nicotinic acid; D-biotin;

p-aminobenzoic acid; folic acid; and inositol. Choline chloride was included in each vitamin mixture, but organisms were not tested for their requirements of this substance. These vitamin mixtures were arranged so that, when a 1 ml aliquot was added to 4 ml of basal medium, their final concentrations were the same as those used by SHANKMAN *et al.* (1947). The final pH of the basal medium/vitamin mixture was 5.2.

All test media were sterilised by subjecting them momentarily to a pressure of 15 lb. sq. inch in an autoclave, then allowing them to cool. Duplicate tubes were inoculated with single drops of a suspension prepared from a 4-5 day old culture grown in apple juice yeast extract medium, from which the organisms were centrifuged, then washed twice in saline and finally resuspended in 15 ml of this solution. Tests were incubated anaerobically at 25°C., the turbidity measured on an 'Eel' nephelometer and the results recorded after 3 or 7 days' incubation, according to the speed of growth of the individual organisms.

Turbidity measurements were made after the nephelometer had been set to a value of 50 units with 5 ml of the standard suspension provided with the instrument. The amounts of growth obtained in the vitamin deficient media were expressed as percentages of the growth occurring in the corresponding complete medium.

RESULTS.

Most of the organisms listed in Table 1 have been described in detail elsewhere (CARR, 1957), but some are included whose characteristics have not been previously described. These include several homofermentative lactic acid bacteria which appear to have properties intermediate between *Lactobacillus plantarum* and *Lactobacillus casei* and which have all been designated homofermentative *Lactobacillus* spec. in Table 1. In addition, there is a single unidentified heterofermentative lactobacillus. There are also three separate groups of cocci; all designated *Leuconostoc mesenteroides*. The two groups called *Leuc. mesenteroides* morphotypes I or II have been previously described. The third group, labelled simply *Leuc. mesenteroides*, shows a range of cultural, morphological and physiological characters intermediate between those of morphotypes I and II.

The vitamin requirements have been classified into three categories: (1) where growth was reduced to 33 percent or less of the

complete control, the omitted vitamin was classed as essential (E); (2) where growth was reduced to between 34 and 66 percent of the complete control, the omitted vitamin was classed as stimulatory (S); (3) in tubes which attained 67 percent or more of the complete control, the omitted vitamin was classed as non-essential (N).

The organisms in Table 1 are arranged in descending order of their vitamin requirements and the vitamins in order of their relative effect. Thus, *L. plantarum* 28 had a complete requirement for five vitamins, whilst at the other extreme *L. pastorianus* 121 had a requirement for only two vitamins.

All organisms had an absolute requirement for calcium pantothenate and all but one required nicotinic acid. Of the remaining vitamins, riboflavin was essential for the growth of more organisms than thiamine, although the total number of organisms wholly dependent on, or stimulated by the latter was considerably higher. It may be noted that the requirements for riboflavin were almost entirely restricted to homofermentative organisms and *Leuconostoc* spec. described as morphotype I. In contrast, only the heterofermentative rods showed a complete requirement for thiamine, and biotin was only essential for five of the organisms tested. Of these, three were homofermentative and the remaining two heterofermentative. Both these organisms showed marked differences from the other heterofermentative organisms as one was a slime-forming strain and the second, unlike any other organism tested, did not require nicotinic acid. Biotin inhibited the growth of *Leuconostoc mesenteroides* 5, which showed 17 percent more growth in the absence of this substance than in a complete vitamin mixture. Vitamin B₆ was wholly required by four homofermentative organisms, folic acid was stimulatory for a single strain of *Leuconostoc* while p-aminobenzoic acid and inositol (not tabulated) were required by none.

DISCUSSION.

The preceding results agree with those of SHANKMAN *et al.* (1947); SNELL (1948); COSTILOW and FABIAN (1954); RUSSELL, BHANDARI and WALKER (1954) and MOORE and RAINBOW (1955). All of these results suggested that pantothenate is required universally by lactic acid bacteria. These workers have shown further that nicotinic acid ranks second in importance to pantothenate as a nutrient for lactic acid bacteria from varying habitats.

Although, in this work, the standards for judging these organisms'

TABLE 1.
Vitamin requirements of lactic acid bacteria from ciders.

Organisms	Code Number	Calcium pantothenate	Nicotinic acid	Riboflavin	Thiamine	Biotin	B ₆	Folic acid
<i>L. plantarum</i>	28	E	E	E	N	E	E	N
Hom. <i>Lactobacillus</i> spec.	40	E	E	E	N	E	E	N
Hom. <i>Lactobacillus</i> spec. (catalase +)	24	E	E	E	N	E	N	N
<i>L. pastorianus</i> (slime prod. +)	34	E	E	N	E	E	N	N
Hom. <i>Lactobacillus</i> spec.	46	E	E	S	N	S	E	N
<i>Leuc. mesenteroides</i> Morph. I	1	E	E	E	S	N	N	N
<i>Leuc. mesenteroides</i> Morph. I	3	E	E	E	S	N	N	N
<i>Leuc. mesenteroides</i> Morph. I	4	E	E	E	S	N	N	N
<i>Leuc. mesenteroides</i> Morph. I	5	E	E	E	S	N	N	N
<i>Leuc. mesenteroides</i> Morph. I	8	E	E	E	S	N	N	N
<i>Leuc. mesenteroides</i> Morph. I	20	E	E	E	S	N	N	N
Hom. <i>Lactobacillus</i> spec.	29	E	E	N	N	S	E	N
<i>L. pastorianus</i>	11	E	E	N	E	N	N	N
<i>L. pastorianus</i>	21	E	E	N	E	N	N	N
<i>Leuc. mesenteroides</i>	25	E	E	E	N	N	N	N
<i>L. pastorianus</i>	39	E	E	N	E	N	N	N
<i>L. pastorianus</i>	53	E	E	N	E	N	N	N
<i>Leuc. mesenteroides</i> Morph. I	9	E	E	S	S	N	N	S
<i>Leuc. mesenteroides</i> Morph. II	12	E	E	N	S	N	S	N
<i>Leuc. mesenteroides</i>	57	E	E	N	S	N	S	N
Het. <i>Lactobacillus</i> spec.	72	E	N	S	S	E	N	N
<i>L. pastorianus</i>	73	E	E	N	S	S	N	N
<i>L. pastorianus</i>	74	E	E	S	S	N	N	N
<i>L. pastorianus</i>	2	E	E	N	S	N	N	N
<i>Leuc. mesenteroides</i> Morph. I	14	E	E	S	N	N	N	N
<i>Leuc. mesenteroides</i>	15	E	E	S	N	N	N	N
<i>Leuc. mesenteroides</i> Morph. I	17	E	E	N	S	N	N	N
<i>Leuc. mesenteroides</i> Morph. II	22	E	E	N	S	N	N	N
<i>L. pastorianus</i>	30	E	E	N	S	N	N	N
Hom. <i>Lactobacillus</i> spec.	33	E	E	N	S	N	N	N
<i>L. pastorianus</i>	45	E	E	N	S	N	N	N
<i>Leuc. mesenteroides</i> Morph. II	60	E	E	N	S	N	N	N
<i>Leuc. mesenteroides</i> Morph. II	62	E	E	N	S	N	N	N
<i>Leuc. mesenteroides</i> Morph. II	68	E	E	N	S	N	N	N
<i>L. pastorianus</i>	105	E	E	N	S	N	N	N
<i>L. pastorianus</i>	109	E	E	N	S	N	N	N
<i>L. pastorianus</i>	121	E	E	N	S	N	N	N

vitamin requirements differed from those used by RUSSELL, BHANDARI and WALKER (1954), it is interesting to note that pantothenate, nicotinic acid, riboflavin and thiamine appear in the same order of importance. The most interesting of these is thiamine since twenty nine of the cider organisms showed some requirement for this vitamin while only three of the organisms examined by RUSSELL, BHANDARI and WALKER (1954) showed any requirement for it. They reported, however, that thiamine had a stimulatory effect on some organisms listed as not requiring this factor, if it was incorporated in a mixture of basal medium and 'essential vitamins'. These results suggest that this vitamin was in fact stimulatory for a wider range of organisms than recorded by these workers, but its role as such was obscured by their more stringent method of scoring vitamin requirements.

It may be that biotin was not required by more organisms because its effect was obscured by the presence of Tween 80 in the medium. WILLIAMS, BROQUIST and SNELL (1947) showed that this substance can replace oleic acid which becomes essential when biotin is omitted. The role of biotin as an inhibitor of some lactic acid bacteria has been previously reported by WHITESIDE-CARLSON and CARLSON (1949) who showed that several vitamins, including biotin, inhibited certain species of *Leuconostoc*.

Similar masking effects might account for the relatively small numbers of organisms requiring vitamin B₆, folic acid and p-aminobenzoic acid. SNELL (1948) has shown with at least one *Lactobacillus* species, namely *L. arabinosus* 17-5, that the requirement for B₆ varies with the composition of the medium. In the same way, he has shown that the requirement for p-aminobenzoic acid and folic acid can be complementary and the requirement for either of these can be masked in the presence of purine bases.

The exacting nutritional requirements of these lactic acid bacteria must play a very important role in controlling their growth in fermenting ciders, their natural habitat. Most juices derived from cider apples have a low nutritional status for micro-organisms and in some it is so poor that yeast fermentation can lead to considerable depletion of thiamine (BURROUGHS and POLLARD, 1953). Further, BURROUGHS and CARR (1956) have shown that the greatest growth of lactic acid bacteria can occur in the juice before the onset of fermentation and in the dry cider after the yeasts have ceased activity and are autolysing. This would suggest that during fermentation

nutrients essential for the full growth of these bacteria are removed. It has been shown that some of these substances are amino acids, but there seems little doubt that B group vitamins also play a part in controlling the growth of lactic acid bacteria during yeast fermentation. In support of this, CASTOR (1953) reported that pantothenic acid, biotin and vitamin B₆ are removed by yeast during the fermentation of grape musts. Thus, in a poor medium, such as cider, the faster growing yeasts will tend to suppress the bacteria which are then only able to grow after yeast activity has ceased and essential nutrients are released from the dead yeast cells. At this time the most suitable residual substrates for the growth of the lactic acid bacteria are traces of non-fermentable sugars, such as pentoses, and organic acids. Of these, the most abundant is malic acid, which is readily metabolised by most of the organisms listed in Table 1, thus giving rise to the well known malo-lactic fermentation.

Acknowledgments.

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A STANDARDIZED ROUTINE COMPLEMENT FIXATION TEST

by

J. H. DE BRUIJN

(Received September 13, 1957).

INTRODUCTION.

Among the great number of complement fixation techniques, that of KOLMER (1929) is preferred by most routine laboratories all over the world. Since the development of the Kolmer test, however, much progress has been made in the study of the immune-hemolytic process, resulting in refined methods of complement and hemolysin titration, which allow a better standardization of test conditions. Some of the factors improving the reproducibility of a complement fixation test are:

1. The titration of complement in terms of 50% hemolytic units (WADSWORTH, MALTANER and MALTANER, 1931), simplified by the application of spectrophotometric measurements (MAYER, EATON and HEIDELBERGER, 1946; KENT, BUKANTZ and REIN, 1946).

2. The addition of magnesium and calcium ions to the diluent, as both play an essential part in the hemolytic process (MAYER, OSLER, BIER and HEIDELBERGER, 1946).

3. The use of an optimal hemolysin level (KENT, 1946), making test results independent of antibodies to sheep erythrocytes which may be present in the serum under examination.

In consequence, several complement fixation tests in which the new principles are applied were elaborated. In routine laboratories, however, substituting one serological test for another, always involves practical difficulties. Moreover, especially in the field of the serodiagnosis of syphilis, the interavailability of test results on national and international level is of the utmost importance. Therefore, a simple, highly standardized, routine complement

fixation technique was developed, which equals the Kolmer test in sensitivity. The present paper deals with the application of the new test in the serodiagnosis of syphilis, employing cardiolipin antigen.

MATERIALS.

Diluent.

All reagents are diluted with veronal-buffered saline (pH 7.4) containing optimal amounts of magnesium and calcium ions. A stock buffer solution is prepared by dissolving 3.00 g 5,5-diethylbarbituric acid in boiling distilled water and adding this solution, after it has been cooled to room temperature, to another, containing 42.50 g sodium chloride and 1.30 g sodium 5,5-diethylbarbiturate. Finally, 5 ml of a solution containing 10.0 g magnesium chloride ($\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$) and 3.3 g calcium chloride ($\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$) per 100 ml is added and distilled water to make 1 l. Before use, 1 volume of this stock solution is diluted to isotonicity by the addition of 4 volumes of distilled water.

Sheep blood.

Sheep blood is aseptically collected in an equal volume of modified Alsever's solution at room temperature and kept at 5°C. (BUKANTZ, REIN and KENT, 1946; BOYDEN, 1951).

Preparation of modified Alsever's solution.

Glucose 2.05 g, sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2 \text{H}_2\text{O}$) 0.80 g and sodium chloride 0.42 g are dissolved in and made up to 100 ml with distilled water. This solution is adjusted to pH 6.1 with a 10% citric acid solution and sterilized at 100° C. during 1 hour.

Hemolysin.

Rabbits are immunized with sheep blood preferably according to the schedule given by DARTER (1953). The serum obtained is heated in a 56°C. waterbath for 30 minutes and preserved with "Merthiolate" (1 : 10000). The optimal dilution of the hemolysin is determined according to the principle of KENT (1946) by estimating the amount beyond which a further increase fails to enhance the hemolytic activity of complement.

Complement.

The serum obtained from a number of healthy guinea-pigs, males

or non-pregnant females, is preserved according to Richardson's method (MACKIE and McCARTNEY, 1950).

Procedure.

Two stock solutions, which keep indefinitely, are prepared in a sodium chloride solution containing 30.6 g per 100 ml:

1. Sodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$) 0.57 g and sodium azide 0.81 g are dissolved and made up to 100 ml.
2. Boric acid 0.93 g, sodium tetraborate 2.29 g and sorbitol 11.47 g are dissolved and made up to 100 ml.

With continuous stirring, 8 parts of serum are mixed with 1 part of solution 1, followed by 1 part of solution 2. The preserved serum should preferably be kept at 5°C. Before use, 1 part of preserved serum is diluted with 7 parts of distilled water. Thus a 1:10 complement dilution is obtained. Any further dilution is made with the diluent. Diluted serum should be used immediately.

Antigen.

The antigen composition originally advocated by MALTANER and MALTANER (1945) and applied to the Kolmer test by KENT, BOYD and SANDERS (1948) was chosen: 0.0175% cardiolipin, 0.0875% lecithin and 0.3% cholesterol in absolute alcohol. Its optimal dilution was established at 1:200 by repeated titrations with syphilitic sera. In preparing this standard dilution, the required amounts of diluent and antigen are measured into separate beakers. The diluent is added rapidly to the antigen and mixed thoroughly by pouring from one beaker into the other and back six times.

METHODS.

Preparation of sensitized erythrocyte-suspension.

An adequate quantity of preserved sheep blood is filtered through gauze and centrifuged. The supernatant fluid and the upper white cell layer are removed by suction through a capillary pipette. Tubes are filled with diluent, the erythrocytes are resuspended by inverting and gently shaking and centrifuged. This washing-process is repeated twice. The volume of the packed erythrocytes is estimated and a preliminary suspension is prepared by adding 40 volumes of diluent.

A 1 ml sample of the suspension is diluted with 9 ml of 0.1% sodium carbonate (Na_2CO_3) solution and the optical density of the lysate is measured against the diluent in a Coleman junior spectrophotometer in a 12 × 75 mm tube at a wavelength of 545 mμ. The suspension is adjusted by adding a volume of diluent, determined according to the formula:

Volume of erythrocyte-suspension to be adjusted = V ml,
optical density measured = x,

$$\text{volume of diluent to be added} = \frac{x - 0.30}{0.30} V \text{ ml.}$$

Before use, the erythrocyte-suspension is sensitized by adding an equal volume of the optimal hemolysin dilution and mixing thoroughly by six successive pourings.

Titration of complement.

Titration is carried out in 12 × 75 mm tubes. Reagents are added according to table 1. Diluent and complement should be mixed thoroughly before adding sensitized erythrocyte-suspension. Tubes are capped and incubated in a 37°C. waterbath for 30 minutes after gently but thoroughly mixing the contents of each tube. This should be repeated when half the incubation time has elapsed.

TABLE 1.
Titration of complement.

Tube number	1	2	3	4	5	6	7	8	9
Diluent, ml	2.00	1.60	1.50	1.40	1.30	1.20	1.50	1.50	1.50
Complement (1 : 50), ml							0.50	0.50	0.50
Complement (1 : 250), ml		0.40	0.50	0.60	0.70	0.80			
Sensitized erythrocyte suspension, ml	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

TABLE 2.
Example of titration of complement.

Tube number	1	2	3	4	5	6	7	8	9
Optical density measured	ref. 0	0.028	0.127	0.271	0.381	0.446	0.491	0.489	0.491
Percentage hemolysis		6	26	55	78	91	mean: 0.490		

Tubes are centrifuged and the optical densities of the supernatant fluids of tubes 2 – 9 are determined spectrophotometrically, using that of tube 1 as reference zero. The percentage hemolysis resulting from each quantity of complement in tubes 2 – 6 is obtained by dividing the optical density of the supernatant fluid of the corresponding tube by the mean value obtained with tubes 7 – 9, and multiplying by 100. The percentages hemolysis, preferably those lying between 20 and 80, are plotted against the corresponding

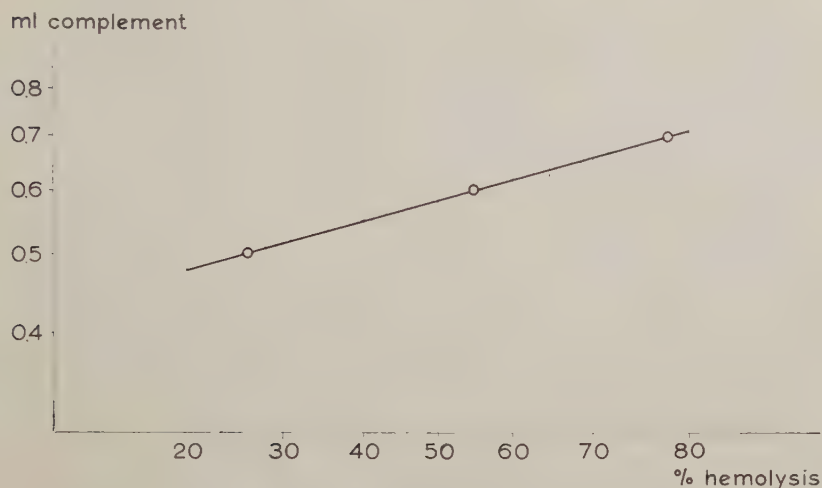


Fig. 1. Graphic determination of K_0 .

quantity of complement on probit-logarithmic ruled paper. A straight line is fitted to the plotted points. A typical titration is presented in table 2 and figure 1. The unit of complement (K_0), i.e. the number of ml of complement (1 : 250) giving 50% hemolysis, is read from the graph and converted, with the help of table 3, into the dilution of complement containing 10 units per ml. In the example given, $K_0 = 0.58$ and the corresponding complement dilution 1 : 43.1.

Preparation of specimens.

Sera and cerebrospinal fluids are heated in a 56°C. waterbath for 30 and 15 minutes respectively.

Performance of the test proper.

The quantitative complement fixation test with serum or cerebrospinal fluid is carried out according to table 4.

In the case of qualitative testing, the scheme is simplified in that only tubes 1 and 2 are used and results, judged with the naked eye, are reported,

if tube 1 shows less than 100% hemolysis, as **anticomplementary**,

if tube 2 shows 100% hemolysis, as **non-reactive**,

if tube 2 shows between 50 and 100% hemolysis, as **inconclusive** and

TABLE 3.

Conversion of K_0 into the dilution of complement containing 10 units per ml.

K_0	Dilution 1:	K_0	Dilution 1:	K_0	Dilution 1:
0.40	62.5	0.54	46.3	0.68	36.8
0.41	61.0	0.55	45.5	0.69	36.2
0.42	59.5	0.56	44.6		
0.43	58.1	0.57	43.9	0.70	35.7
0.44	56.8	0.58	43.1	0.71	35.2
0.45	55.6	0.59	42.4	0.72	34.7
0.46	54.3			0.73	34.2
0.47	53.2	0.60	41.7	0.74	33.8
0.48	52.1	0.61	41.0	0.75	33.3
0.49	51.0	0.62	40.3	0.76	32.9
		0.63	39.7	0.77	32.5
0.50	50.0	0.64	39.1	0.78	32.1
0.51	49.0	0.65	38.5	0.79	31.6
0.52	48.1	0.66	37.9		
0.53	47.2	0.67	37.3	0.80	31.3

TABLE 4.

Complement fixation test.

Tube number	1	2	3	4	5	
Specimen dilution		1 : 1	1 : 2	1 : 4	1 : 8	
Diluent, ml	0.25		0.25	0.25	0.25	
Serum or cerebrospinal fluid, ml	0.25	0.25	0.25			
			L	0.25		
				L	0.25	
					L	0.25
Antigen (1 : 200), ml		0.25	0.25	0.25	0.25	

The contents of each tube are mixed thoroughly.

Complement (10 units/ml), ml | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |

The contents of each tube are mixed thoroughly and fixation is allowed to proceed for 16 hours in a 5°C. refrigerator and for 15 minutes in a 37°C. waterbath.

Sensitized erythrocyte suspension, ml | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |

The contents of each tube are mixed thoroughly and hemolysis is allowed to proceed for 30 minutes in a 37°C. waterbath.

if tube 2 shows less than 50% hemolysis, as r e a c t i v e.

In the case of quantitative testing, results are reported in terms of t i t e r, the titer being defined as the highest reactive specimen dilution.

S u m m a r y.

A description is presented of a simple routine complement fixation test in which veronal-buffered saline, containing magnesium and calcium ions, is used as a diluent and an optimal hemolysin dilution and preserved complement, spectrophotometrically titrated in terms of 50% hemolytic units, are applied.

A c k n o w l e d g e m e n t.

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SOME DATA ON THE OCCURRENCE OF *SALMONELLA* IN ANIMALS IN SURINAM

by

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(Received September 24, 1957).

INTRODUCTION.

During an investigation into the occurrence of *Leptospira* in Surinam, advantage was taken of the opportunity thus presented to utilize the available material to gain a general impression of the extent to which *Salmonella* carriers existed among various species of animals met with there.

Very few data on isolations of *Salmonella* from man and animals in Surinam are available; except for the records in the annual reports of the Medical Service of the regular occurrence of a number of *Salmonella typhi*-infections in man, the only other reports deal with *S. gallinarum-pullorum* in poultry (FRICKERS, 1939, LANGELEER, 1956).

For a general survey of the *Salmonella* types isolated in the Caribbean area the reader is referred, inter alia, to the studies made by GUILBRIDE (1953) and by SUTMÖLLER and KAMPELMACHER (1957).

CULTURAL TECHNIQUE.

The investigation embraced cattle, pigs, dogs, rats (*Rattus norvegicus*), awaris (*Didelphis marsupialis marsupialis* and *Caluromys philander*), fowl and toads (*Bufo marinus*). With the exception of the fowl, none of the animals examined showed any clinical symptoms of salmonellosis.

¹) Director: Prof. Dr F. C. KRANEVELD.

²) Head: Dr A. CLARENBURG.

The cattle and pig material was obtained from animals slaughtered in Paramaribo abattoir ¹⁾, that of the dogs from stray dogs found in this town, while the rats and awaris were caught in traps in Paramaribo or in the immediate vicinity of the town.

The choice of the actual material used for the examination was governed by the species of animal in question. In the case of cattle bile, portal and mesenteric lymphatic glands, and three sections of the intestine, *viz.*, the beginning of the duodenum, the ileocecal valve and the rectum were examined. For pigs and dogs material was used from the mesenteric lymphatic glands, the beginning of the duodenum, the ileocecal valve and the rectum, while it was limited to the small intestine in the examination of toads. In addition to the intestinal contents, the bacteriological examination of fowl was also extended to the spleen and the liver.

The material under investigation was first inoculated in tetrathionate broth (Difco); after incubation for 20 hours at 37°C. 1 or 2 brilliant green agar plates (Difco) ²⁾ were inoculated. It was necessary to limit the investigation to one sub-culture of a suspect *Salmonella* colony, this being subsequently examined with a polyvalent agglutinating O-serum. If no agglutination occurred the morphology of the bacterium, the motility and the behaviour of the organism in glucose and lactose broth were studied. When the characteristics were found to correspond with those of *Salmonella*, as also in the case of *Salmonella* colonies evidencing a positive agglutination reaction, a sub-culture was prepared in serum-agar and forwarded to the National Salmonella Centre, National Institute of Public Health, Utrecht (The Netherlands) for further differentiation. The antigenic structure was investigated there according to the KAUFFMANN-WHITE-scheme.

Lymphatic gland material was examined by incorporating finely ground tissue in tetrathionate broth. The bile was directly inoculated on brilliant green agar plates ²⁾.

Intestinal material was collected by means of sterile swabs

¹⁾ We would like to take this opportunity of extending our thanks to the Head of the Veterinary Service, W. J. C. REININGH, for his kind assistance and co-operation.

²⁾ Instead of brilliant green-agar plates, desoxycholate citrate lactose sucrose plates (Baltimore Biological Laboratories) were used for 67 of the 118 cattle specimens examined.

which, after having been in contact with the intestinal mucosa and intestinal contents at the desired area, were immersed in the tetrathionate broth and the wooden swab holder broken off at the edge of the tube.

The results of our investigations are summarized in tables 1 and 2.

TABLE 1.
The occurrence of *Salmonella*-types in animals in Surinam.

	Ox	Pig	Dog	Rat	Awari	Toad	Hen
<i>S. tinda</i>		+					
<i>S. paratyphi</i> B				+			
<i>S. reading</i>		+	+				
<i>S. kaapstad</i>						+	
<i>S. san diego</i>					+	+	
<i>S. typhi murium</i>				+			
<i>S. oranienburg</i>					+		
<i>S. newport</i>			+		+	+	
<i>S. glostrup</i>			+				
<i>S. wuerzburg</i>		+	+	+	+		
<i>S. enteritidis</i> var. <i>danyisz</i>				+			
<i>S. dublin</i>							+
<i>S. panama</i>	+			+	+	+	
<i>S. gallinarum-pullorum</i>							+
<i>S. newington</i>							+
<i>S. abacetuba</i>				+	+	+	
<i>S. rubislaw</i>				+	+	+	
<i>S. maelia</i>				+			

DISCUSSION.

Our findings serve to emphasize the great value of collecting specimens for examination from various parts of the intestine, as the presence of *Salmonella* was only detected in the majority of cases in the nutrient media inoculated with material from one of the intestinal sections examined, i.e. the reaction was not positive for all the intestinal sections of a given animal. This is probably best exemplified by the fact that, of the 36 "positive" animals from which cultures had been obtained from 2 or 3 parts of the intestine, there were only 6 cases in which a positive result was obtained simultaneously at 2 different points in the intestine. In two of these animals 2 *Salmonella* types were found in the various intestinal sections.

TABLE 2.

Animal	No. of animals examined	No. reacting positively	<i>Salmonella</i> types
Ox	118	1	<i>S. panama</i>
Pig	93	3	<i>S. reading</i> <i>S. tinda</i> <i>S. wuerzburg</i>
Dog	84	5	<i>S. newport</i> (2×) <i>S. glostrup</i> <i>S. reading</i> <i>S. wuerzburg</i>
Awari	60	16	<i>S. panama</i> (8×) <i>S. abaetetuba</i> (2×) <i>S. newport</i> (2×) <i>S. oranienburg</i> <i>S. rubislaw</i> <i>S. san diego</i> <i>S. wuerzburg</i>
Rat	168	13	<i>S. panama</i> (3×) <i>S. enteritidis</i> var. <i>danyisz</i> (2×) <i>S. abaetetuba</i> (2×) <i>S. wuerzburg</i> (2×) <i>S. maderia</i> <i>S. paratyphi</i> B <i>S. rubislaw</i> <i>S. typhi murium</i>
Toad	27	15	<i>S. newport</i> (6×) <i>S. abaetetuba</i> (3×) <i>S. panama</i> (2×) <i>S. rubislaw</i> (2×) <i>S. kaapstad</i> <i>S. san diego</i>
Fowl ¹⁾	25	9	<i>S. gallinarum-pullorum</i> (7×) <i>S. dublin</i> <i>S. newington</i>

¹⁾ These were either diseased or dead birds.

SUTMÖLLER and KAMPELMACHER (1957) have drawn attention to the potential danger to public health attending the cattle imported into Aruba for slaughter, a high percentage of which appear to be infected with *Salmonella*, unless effective control is exercised. A comparison with the available data in respect of the Netherland Antilles shows that the number of *Salmonella* carriers among the population (private communication, W. A. COLLIER) — if *S. typhi* is disregarded — livestock and domestic animals in Surinam is comparatively small. Whereas conditions in the Netherland Antilles render the continual import of *Salmonella* carriers unavoidable in practice, Surinam on the other hand may be regarded as a more or less closed area. Indigenous cattle in Surinam (very few goats and sheep are slaughtered, e.g. the total did not exceed 153 in 1953) are kept extensively, so that the chances of faecal contact are fairly slight; in addition, lowered resistance during transportation to the slaughter-house is likewise a factor of relatively small importance in view of the short distances involved. The most plausible reason for the comparatively low degree of *Salmonella* infection of man, livestock and other domestic animals in Surinam would appear to be the fact that there is no need to import livestock for slaughter. On the other hand, the high percentage of non-domestic animals (awaris, rats and toads) infected with *Salmonellae* can be explained by their frequent contact with infected material.

S u m m a r y.

During an investigation in Surinam 18 *Salmonella* types were isolated from material obtained from cattle, pigs, dogs, rats, awaris, fowl and toads.

A c k n o w l e d g e m e n t.

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THE EFFECT OF NITRITE ON NITRATASE ADAPTATION IN *E. COLI*

by

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During experiments on the nitrate-nitrite relationships of a *Bacillus* spec. isolated in this laboratory and described elsewhere (EDDY and INGRAM, 1956), results were obtained with cultures growing at pH 5.5 which could be explained on the basis of the inhibition of nitratase formation by nitrite at concentrations of the order of 0.0015 - 0.004 M. This could not be pursued, however, because this organism produced so active a nitritase that nitratase could not be estimated by following nitrite accumulation, while methods of nitrate estimation are inadequate for measuring rates of nitrate disappearance. In view of this difficulty it was decided to investigate the possible effects of nitrite on nitratase adaptation in *Escherichia coli* for, although POLLOCK (1946) reported that 0.001 M nitrite was without effect, the concentrations involved here were somewhat higher.

METHODS.

O r g a n i s m. Overnight cultures of *E. coli* (EDDY, 1952) grown on digest agar (McCartney) in Roux bottles at 37° were harvested and washed twice with Ringer solution (quarter strength). A suspension containing approximately 1.5×10^{11} cells/ml (about 6 mg N/ml) was prepared and stored at +5°.

N i t r a t a s e a d a p t a t i o n. The adaptation technique was essentially the same as that described by POLLOCK (1946). Thunberg tubes containing 1 ml suspension, 2 ml 0.2 M mannitol, 2 ml 0.2 M

KNO₃, and 5 ml buffer were evacuated and incubated in a water-bath at 37°. The buffers used were 0.4 M Na₂HPO₄/KH₂PO₄ at pH 7.2 and 6.5 and 0.6 M Na₂HPO₄ + 0.3 M citric acid at pH 5.8.

In some experiments 1 ml of the buffer was replaced by 1 ml 2% (w/v) casein hydrolysate (Oxoid). Nitratase adaptation is considerably enhanced by the presence of amino acids in the adaptation mixture (POLLOCK and WAINWRIGHT, 1948) and will even take place in the absence of nitrate in the presence of high concentrations of amino acids (WAINWRIGHT, 1950). In most of the experiments here the latter type of nitratase formation was minimized by the use of heavy suspensions and relatively low concentrations of amino acids.

After incubation, the tubes were shaken and opened and 7.5 ml of the contents transferred to a centrifuge tube. The cells were washed twice with half-strength buffer to free them from nitrite and then suspended to a volume of 1.5 ml i.e. half the density of the stock suspension.

Nitratase estimation. Nitratase activity was measured by the production of nitrite under standard conditions. Since it was desired to measure the nitratase activities of cells after adaptation under different conditions, it was essential that no further adaptation should take place during the estimation. In addition, the simultaneous reduction of nitrite, which occurred more rapidly with nitrate-adapted than with unadapted cells, had to be avoided because it otherwise gave results which were too low. Hence, formate was used as H-donor because it does not serve as a C-source for adaptation (POLLOCK, 1946) and dinitrophenol was added to inhibit nitrite reduction (LASCELLES and STILL, 1946). It was subsequently found that formate would not serve as an H-donor for nitrite reduction and that dinitrophenol prevented adaptation to nitrate.

Thunberg tubes containing 5 ml of the buffer at pH 7.2, 1 ml 0.2 M sodium formate, 1 ml 0.2 M KNO₃, 2 ml 0.005 M 2:4-dinitrophenol and 1 ml of the adapted suspension were evacuated and incubated in a water-bath at 37°. Nitrite estimations were carried out on samples removed at suitable intervals – usually 30 min. After sampling, the tubes were re-evacuated and replaced in the water-bath. Under these conditions, constant rates of nitrite production were obtained with any particular sample. Throughout this paper the nitratase activity is expressed as Q_{NO_3} , i.e. μ moles NO₃ reduced/mg bacterial N/hr.

Nitrite estimation. The samples were diluted to give

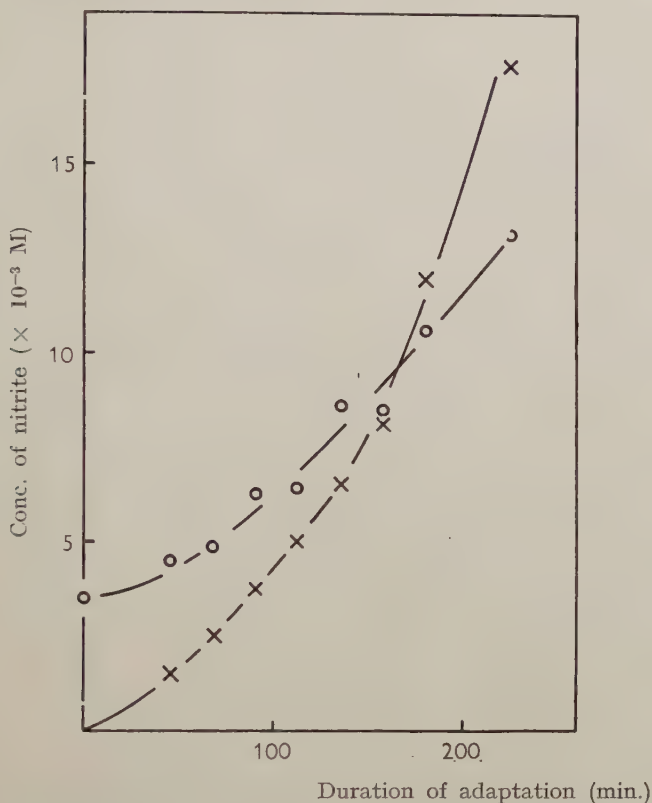


Fig. 1. Accumulation of nitrite during nitratase adaptation by *E. coli* in the initial presence and absence of nitrite. Adaptation mixture as described in the Methods section; pH 7.2; 37°. Amino acids not present.

x—x nitrite absent initially.

o—o 3.6×10^{-3} M nitrite present initially.

a nitrite concentration in the range 0.5–3.5 μ g NaNO_2 /ml. To 10 ml of the diluted sample were added 5 ml of reagent (equal volumes of 0.1 g α -naphthylamine in 150 ml 50% v/v acetic acid and 0.05 g sulphanilic acid in 150 ml 50% acetic acid), the mixture centrifuged for 15 min. to remove bacterial cells and the intensity of colour read in an EEL photoelectric colorimeter using filter 624 (520 $m\mu$). The nitrite concentration was found by reference to a standard calibration curve and then multiplying by the dilution factor.

RESULTS.

Since nitrite accumulates at an accelerating rate during adap-

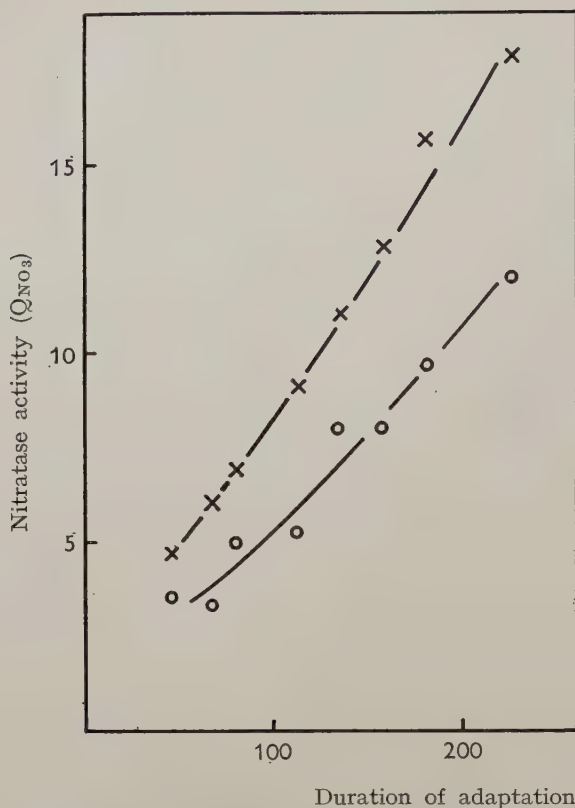


Fig. 2. Increase in nitratase activity during adaptation by *E. coli* in the initial presence and absence of nitrite. Adaptation mixture as described in the Methods section; pH 7.2; 37°. Amino acids not present.
 Q_{NO_3} = μ moles nitrate reduced/mg bacterial N/hr.

x—x nitrite absent initially.

o—o 3.6×10^{-3} M nitrite present initially.

tation and it was not possible to remove this nitrite from the system, adaptation clearly could not be carried out in the absence of nitrite. However, there were two promising methods of deciding whether nitrite is inhibitory. First, a comparison of the rates of nitrite accumulation and nitratase formation in adaptation mixtures with and without nitrite present initially. Second, a comparison of the rates of nitrite accumulation and nitratase formation using two different concentrations of cells since, with lower concentrations, nitrite should accumulate less rapidly and adaptation should therefore go on longer.

Adaptation with nitrite present initially. Four Thunberg tubes

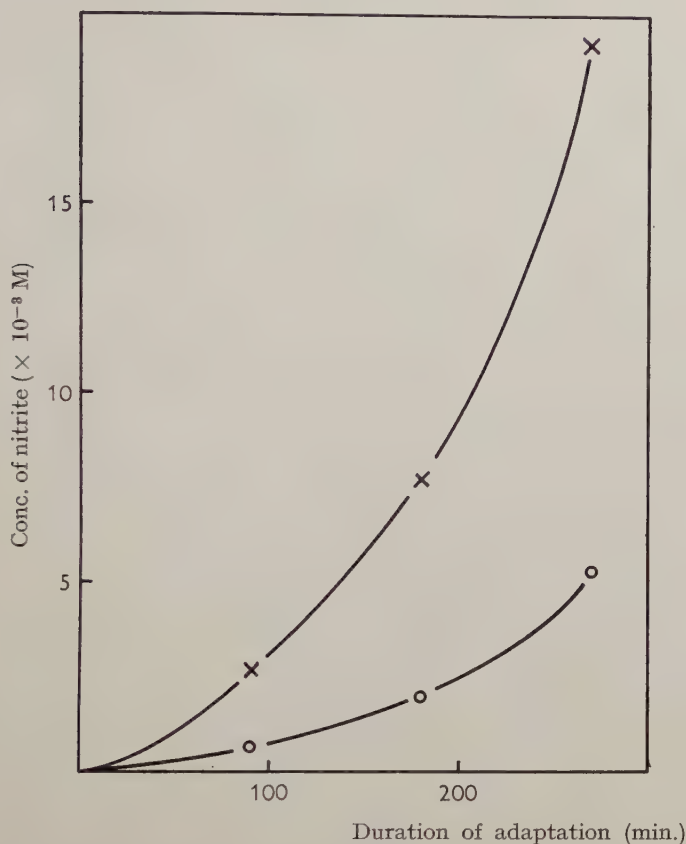


Fig. 3. Accumulation of nitrite during nitratase adaptation by *E. coli* using two concentrations of cells. Adaptation mixture as described in the Methods section; pH 7.2; 37°. Amino acids not present.

x — x 1.5×10^{10} cells/ml.

o — o 7.5×10^9 cells/ml.

containing adaptation mixtures were set up as described in the Methods section, opened serially at intervals and nitrite and nitratase estimations carried out on the contents. The following day the procedure was repeated, the adaptation being stopped at different times. On the two following days the experiment was repeated using adaptation mixtures containing 3.6×10^{-3} M nitrite. Storage of the stock suspension at $+5^\circ$ during the experimental period of 4 days had no effect on its ability to adapt, measured by the accumulation of nitrite during adaptation.

The results are shown in Figs. 1 and 2. The former shows the

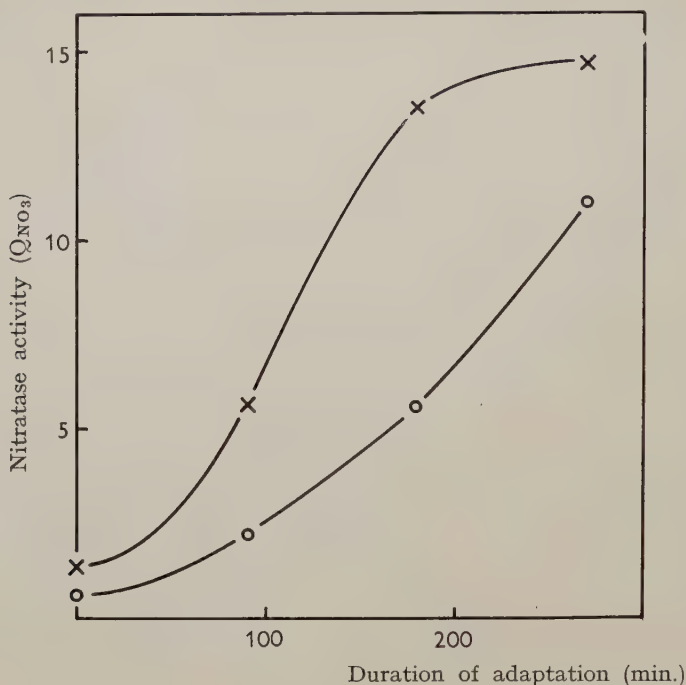


Fig. 4. Increase in nitratase activity during adaptation by *E. coli* using two concentrations of cells. Adaptation mixture as described in the Methods section; pH 7.2; 37°. Amino acids not present. $Q_{NO_3} = \mu$ moles nitrate reduced/mg bacterial N/hr.

x — x 1.5×10^{10} cells/ml.

o — o 7.5×10^9 cells/ml.

accumulation of nitrite during the course of the adaptation, the latter the nitratase activity (Q_{NO_3}) of the suspension after the periods of adaptation indicated. When nitrite was present initially, the rates of nitrite accumulation and nitratase formation were lower than when there was no initial nitrite. The effect on nitrite production was not due to inhibition of nitratase by nitrite since linear rates of nitrate reduction could be maintained to higher concentrations of nitrite than those recorded here.

Use of different cell densities. Nitrite accumulation and nitratase production were followed, as already described, in tubes containing 1 ml and 0.5 ml of suspension. The results, given in Figs. 3 and 4, show that halving the cell density more than halved the rate of nitrite accumulation but that nitratase production was correspondingly lower.

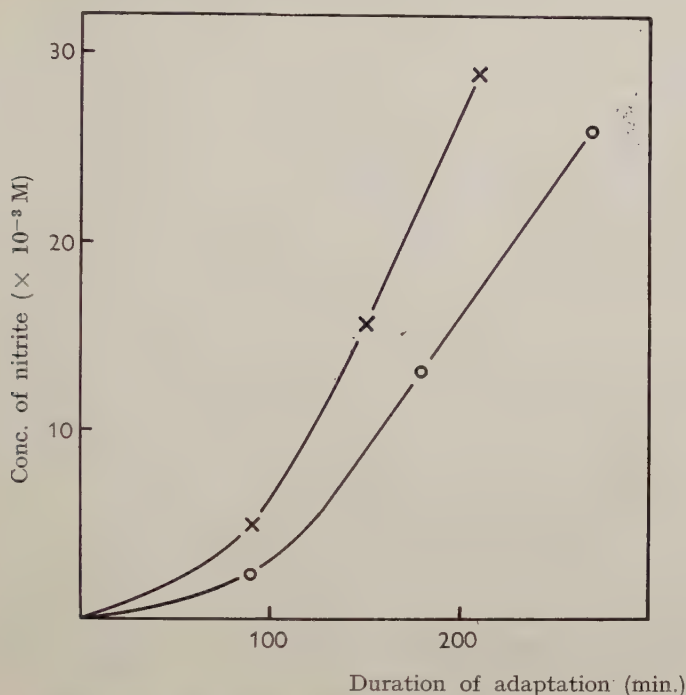


Fig. 5. Accumulation of nitrite during adaptation by *E. coli* using two concentrations of cells. Adaptation mixture as described in the Methods section; pH 7.2; 37°. Amino acids present.

x — x 1.5×10^{10} cells/ml.

o — o 7.5×10^9 cells/ml.

The disproportionate effect of the dilution of bacterial suspensions has been reported for other systems by several workers and for that under investigation by POLLOCK (1946) but the reasons for it are not clearly understood. Since POLLOCK and WAINWRIGHT (1948) found the presence of an external nitrogen supply to be necessary for adaptation in their suspensions, the above experiment was repeated using adaptation mixtures containing 0.2% casein hydrolysate. The effect was very marked (Figs. 5 and 6). Not only was the disproportionate effect of dilution eliminated but the nitratase activities were much higher than those obtained in experiments without casein hydrolysate. The results in Figs. 3-6 show that when, during adaptation, nitrite production was reduced by using fewer cells, adaptation continued for a longer time and more enzyme was ultimately produced per cell.

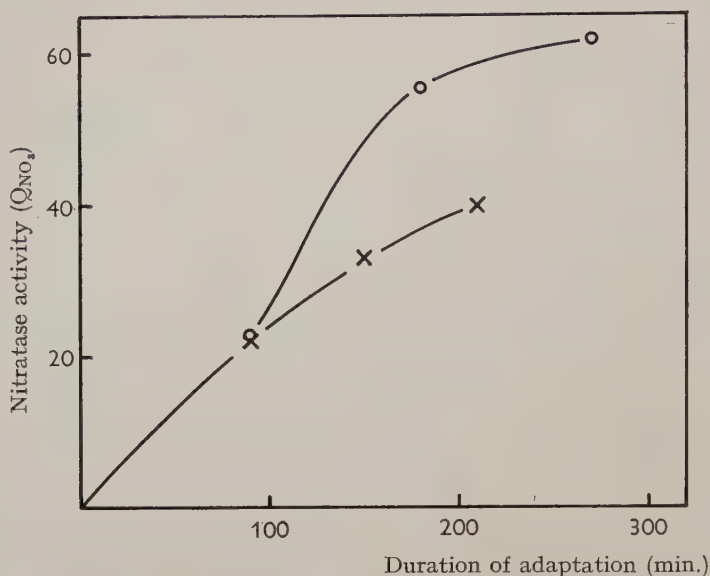


Fig. 6. Increase in nitratase activity during adaptation by *E. coli* using two concentrations of cells. Adaptation mixture as described in the Methods section; pH 7.2; 37°. Amino acids present. Q_{NO_3} = μ moles nitrate reduced/mg bacterial N/hr.

x — x 1.5×10^{10} cells/ml.

o — o 7.5×10^9 cells/ml.

The experiments with nitrite present initially were repeated using adaptation mixtures containing casein hydrolysate and the same pattern was obtained.

Interaction of pH and initial nitrite concentration on nitratase adaptation.

TARR (1941) showed that the bacteriostatic action of sodium nitrite was largely dependent upon the pH of the culture medium and similar findings have been reported by other workers (CASTELLANI and NIVEN, 1955; EDDY and INGRAM, 1956). It was thought that this might be due to increased concentrations of unionized nitrous acid at lower pH values. Therefore the effects of different initial nitrite concentrations on the nitratase production during a given time were compared at different pH values.

First, the effect of pH without initial nitrite was investigated. Adaptation mixtures were prepared, with and without casein hy-

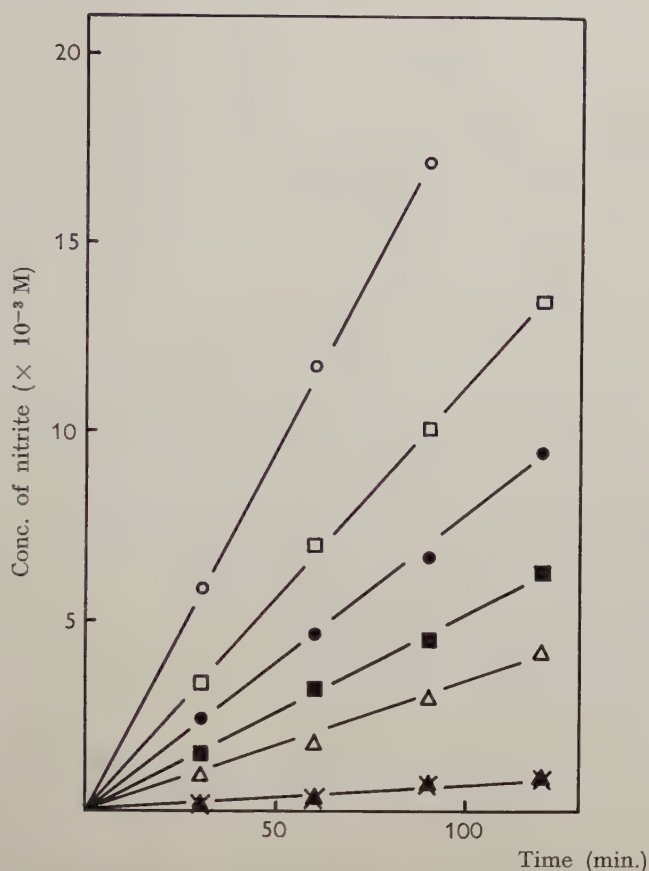


Fig. 7. The production of nitrite from nitrate during estimations of nitratase activity as described in the Methods section; pH 7.2; 37°. Cells previously adapted at pH 7.2, 6.5 or 5.8 with or without amino acids (AA).

- | | |
|-----------------------|-------------------|
| ● — ● pH 7.2 — AA | ○ — ○ pH 7.2 + AA |
| ■ — ■ pH 6.5 — AA | □ — □ pH 6.5 + AA |
| ▲ — ▲ pH 5.8 — AA | △ — △ pH 5.8 + AA |
| x — x unadapted cells | |

drollysate, at pH 7.2, 6.5 and 5.8 and incubated at 37° for 4 hr. The cells were then washed and used for nitratase estimations as already described. The pH was altered only for the adaptation: the nitratase estimations were carried out at pH 7.2. The results are shown in Fig. 7, in which are plotted the rates of nitrite production under standard conditions by the variously adapted cells together with the rate by unadapted cells.

Since there was only a 5-fold increase in activity at pH 5.8, and then only in the presence of casein hydrolysate, it appeared that no useful experiment could be done at this pH with nitrite present initially. Hence subsequent experiments were done at pH 7.2 and 6.5 only. Two further suspensions of *E. coli* were used for the experiments with nitrite present initially and casein hydrolysate was omitted from the adaptation mixtures because nitrous acid reacts with amino groups.

TABLE 1.

Nitratase activities (Q_{NO_3}) of two suspensions of *E. coli* adapted for 4 hours as described in the Methods section with different initial concentrations of nitrite at pH 7.2 and 6.5.

Initial nitrite concentration ($\times 10^{-3}$ M)	pH 7.2			pH 6.5		
	Q_{NO_3}		Percentage of adaptation in absence of initial nitrite	Q_{NO_3}		Percentage of adaptation in absence of initial nitrite
	Suspension 1	Suspension 2		Suspension 1	Suspension 2	
0.0	18.5	24.0	100	10.8	14.3	100
1.45	17.0	—	92	6.8	—	63
2.9	—	19.5	81	—	6.5	45
4.35	10.5	—	57	4.5	—	42
7.25	—	14.0	58	—	3.3	23
10.15	—	10.0	42	—	2.3	16
14.5	6.3	—	34	1.0	—	9.3

Table 1 shows the nitratase activities (Q_{NO_3}) after adaptation for 4 hours with different initial nitrite concentrations at pH 7.2 and 6.5. When the results were plotted, as percentages of the nitratase produced in the initial absence of nitrite, against nitrite concentration (Fig. 8), they fell into two distinct series, one for each pH. This suggests that pH, besides its effect on adaptation already shown, influenced the effect of nitrite on adaptation. Following the suggestions already made that this might be due to the effect of pH on the ionization of nitrous acid the results have been replotted (Fig. 9) as percentage suppression of nitratase adaptation against concentration of unionized nitrous acid (pK of $HNO_2 = 3.4$). On this basis the results approximate much more closely to a single series.

Influence of amino acids on adaptation. Besides abolishing the

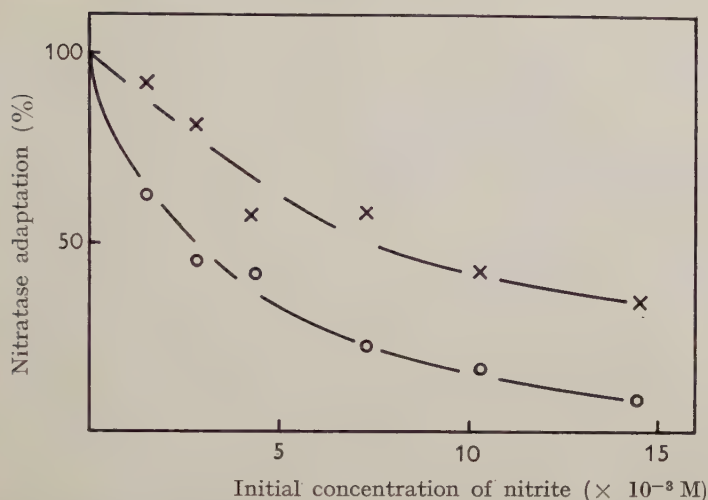


Fig. 8. Relationship between the nitratase produced during 4 hr adaptation and the initial concentration of nitrite in the adaptation mixture.

x — x pH 7.2

o — o pH 6.5

adaptation taking place in the initial
absence of nitrite.

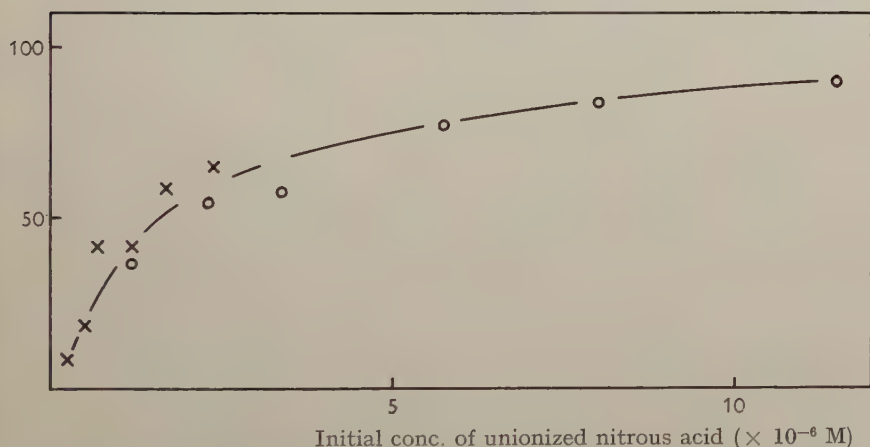


Fig. 9. Relationship between the suppression of nitratase adaptation due to nitrite present initially in the adaptation mixture and the concentration of unionized nitrous acid present initially (pK of $\text{HNO}_2 = 3.4$).

x = experiments carried out at pH 7.2

o = experiments carried out at pH 6.5

dilution effect, amino acids stimulate nitratase adaptation (Fig. 7), particularly at pH 5.8 where no adaptation takes place in the absence of amino acids. This latter action of amino acids is not due to general growth of the cells in suspension, comparable with the increase in nitratase, since there is no measurable increase in the total nitrogen content of the suspensions during adaptation whether or not this takes place in the presence of amino acids. Since nitrous acid reacts with amino acids, the inhibition of nitratase adaptation by the resulting nitrite, and the stimulation of the adaptation by amino acids, suggest that the stimulatory action of amino acids might be due to destruction of nitrous acid. Adaptation mixtures incorporating 0.2% (w/v) casein hydrolysate were prepared at pH 7.2 and 6.5, containing sodium nitrite at concentrations which should give approximately the same degree of inhibition of adaptation at each pH, i.e. 10×10^{-3} M and 3.6×10^{-3} M respectively. After incubation and washing, nitratase estimations were made on these and on control suspensions which had been adapted without casein hydrolysate. If amino acids act by destroying nitrite, the degree of inhibition of adaptation should be less in the presence of amino acids than in their absence. The results (Table 2) were inconclusive at pH 7.2 though there was some difference at pH 6.5. However, it seems clear that the stimulatory action of amino acids cannot be due solely to destruction of nitrous acid since the concentration of nitrite is greater during adaptation in the presence of amino acids than in their absence (cf. Figs. 1 and 5).

Effect of nitrite on growth of *E. coli*. It has been known since the work of QUASTEL, STEPHENSON and WHETHAM (1925) that growth of *E. coli* is inhibited by nitrite and it was therefore desirable that the bacteriostatic effect of nitrite should be investigated under conditions approximating closely to those under which adaptation takes place: in particular in the absence of oxygen. Under comparable conditions there was no growth in broth cultures kept anaerobically. Hence, for this purpose, plates of tryptic digest agar adjusted to pH 6.5 and containing no nitrite and 3.5, 7.0 and 14×10^{-3} M nitrite, were poured, inoculated with spread drops of *E. coli* and incubated in anaerobic jars for 18 hr. The colony diameters were then measured. The results, given in Table 3, show degrees of inhibition comparable with those attained in the adaptation experiments (cf. Table 1).

TABLE 2.

Effect of nitrite and casein hydrolysate, separately and together, upon nitratase adaptation.

	Addition to adaptation mixture	Q_{NO_3}	% Suppression of adaptation
pH 7.2	None	16.5	0
	1×10^{-2} M $NaNO_2$	10.0	40
	0.2% casein hydrolysate	41	0
	0.2% " " + 1×10^{-2} M $NaNO_2$	27	34
pH 6.5	None	11.3	0
	3.6×10^{-3} M $NaNO_2$	6.8	40
	0.2% casein hydrolysate	24	0
	0.2% " " + 3.6×10^{-3} M $NaNO_2$	18	25
pH 5.8	None	1.5	—
	0.2% casein hydrolysate	7.2	—
Unadapted cells		1.6	—

TABLE 3.

Effect of nitrite on the anaerobic growth of *E. coli* on tryptic digest agar at pH 6.5.

Conc. of nitrite ($\times 10^{-3}$ M)	Diameter of colonies (mean of 10 measurements) in mm
0	0.95
3.5	0.84
7.0	0.54
14	0.23

DISCUSSION.

The results show clearly that nitratase adaptation in washed cell suspensions of *E. coli* is inhibited by nitrite. This implies that nitratase adaptation in the presence of nitrate must be auto-inhibitory. As it is impossible to maintain a constant nitrite concentration in the adapting system, it is difficult to define quantitatively the relationship between nitrite concentration and rate of nitratase adaptation. A simple relationship may, in fact, not exist, as comparison of the results of the experiments with initial nitrite and those

using different cell densities suggests. Since nitrite at the concentrations involved does not affect nitratase activity, it is obvious from Figs. 1 and 2 that 3.6×10^{-3} M nitrite affects nitratase adaptation adversely from the very beginning of the experiment. The results in Figs. 5 and 6, however, suggest that the effect of nitrite is not noticeable below about $6-7 \times 10^{-3}$ M. This difference is not due to the presence of amino acids (cf. Fig. 3). It may mean that the rate of nitratase formation is a function of both nitrite concentration and the amount of nitratase already present, for nitratase is very low in unadapted cells but is considerably higher by the time the nitrite concentration in experiments without initial nitrite has reached the initial value in the others.

The effect of pH on this inhibitory action of nitrite has been demonstrated, comparable with that on the bacteriostatic action previously reported (TARR, 1941; CASTELLANI and NIVEN, 1955; EDDY and INGRAM, 1956). The degree of inhibition is more closely related to the concentration of unionized nitrous acid than to the total nitrite present, which may be connected with penetration into the bacterial cell. POLLOCK (1946) concluded that nitrite was without effect on nitratase adaptation probably because the concentrations involved were too low at the relatively high pH he used (7.2).

The close relationship between the nitrate and nitrite ions suggests that the inhibitory effect of nitrite on nitratase adaptation might be competitive. However, the results obtained in the growth experiments where the inhibition paralleled that on adaptation, suggest that the inhibitory action is a general one on protein synthesis.

It has been noted previously that the reasons for the dilution effect observed when comparing adaptation with two concentrations of cells (Figs. 3 and 4) are not clearly understood. The abolition of the effect by amino acids, taken with the evidence presented that the effect of amino acids is not solely due to destruction of nitrous acid, agrees with the earlier suggestion of POLLOCK and WAINWRIGHT (1948) that the stimulatory effect of amino acids may be related to synthetic metabolism.

The work described in this paper was carried out as part of the programme of the Food Investigation Organisation of the Department of Scientific and Industrial Research.

Summary.

1. Nitratase adaptation in *E. coli* is inhibited by ca. 6×10^{-3} M nitrite at pH 7.2.
2. The degree of inhibition is more closely related to the concentration of unionized nitrous acid than to the total nitrite concentration.
3. Since conditions allowing adaptation also favour nitrite production, nitratase adaptation must be auto-inhibitory.
4. The inhibitory effect of nitrite on growth, which parallels that on adaptation, is probably a general one on protein synthesis.
5. The stimulatory action of amino acids on the adaptation is not due to the destruction of nitrite.

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A NEW *SALMONELLA* TYPE (*S. AMSTERDAM*)

by

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(Received February 4, 1958).

In October 1957 a culture was isolated by G. J. P. SCHAAP and P. J. DE VRIES (Municipal Laboratory of Public Health, Amsterdam) from the stool of a 32 years old patient, who was affected with gastroenteritis. The patient was a pilot, who had been during the last time in Montreal, Istanbul, Beirut and Karachi.

The culture showed the morphological and biochemical characteristics of the genus *Salmonella*, e.g. no fermentation of adonitol, inositol, lactose, salicin, sucrose and glycerol. No production of indole, no decomposition of urea and no liquefaction of gelatin. Rapid fermentation of arabinose, dulcitol, glucose (with gas), maltose, mannitol, rhamnose, sorbitol, trehalose and xylose. Positive reaction in Stern's glycerol-fuchsin broth, and formation of H_2S . Positive reaction in d-tartrate and mucate after 1 day, and negative reaction in l- and i-tartrate after 14 days. Nitrates were reduced, the Voges Proskauer reaction was negative and the methyl red reaction was positive. The culture did not grow in KCN substrate.

On serologic examination the organism was found to be a member of O group E (3,10). It was agglutinated to titre by *S. anatum* O serum; in cross absorption tests all antigens were removed. The H antigens were monophasic. The culture was agglutinated to titre by *S. montevideo* H serum; in cross absorption tests all H antigens were removed.

The antigenic formula of the type was therefore 3, 10 : g,m,s:-. As a *Salmonella* type with this formula is as yet not described, we propose the name *Salmonella amsterdam*.

We are indebted to Dr F. KAUFFMANN, International Salmonella Centre (Copenhagen), for the confirmation of our findings.

S u m m a r y.

A new *Salmonella* type, *Salmonella amsterdam*, isolated from the stool of a pilot, was described. The antigenic formula is 3,10 : g,m,s:-.

(Stritch School of Medicine and Graduate School, Loyola University, Chicago and Agricultural Research Service, U.S.D.A., Beltsville, Md.).

FLAGELLAR CHARACTERISTICS OF *RHIZOBIUM* SPECIES

by

EINAR LEIFSON and LEWIS W. ERDMAN

(Received February 4, 1958).

INTRODUCTION.

Because of its economic importance the genus *Rhizobium* has been the subject of numerous publications. The literature up to 1932 has been reviewed in the monograph by FRED, BALDWIN and MCCOY. In this literature two types of flagellation are described in strains of *Rhizobium*, namely monotrichous and peritrichous. Various authors are not always in complete agreement as to the flagellation of the named species. Since 1932 few papers have been published dealing with the flagellation of rhizobia and none have been sufficiently comprehensive to clarify the subject. The paper by CONN and ELROD in 1947 deals with only a few strains from only three plant types and is not sufficiently comprehensive to have general significance. The papers by BISSET and HALE in 1951, and BISSET in 1952 on life cycles in rhizobia require considerable confirmation.

The purpose of the present paper is to describe and illustrate the flagellation, as observed on laboratory media, of strains of *Rhizobium* from a comprehensive number of plant species.

MATERIALS AND METHODS.

The 82 cultures studied were selected from the extensive collection of *Rhizobium* strains maintained in the laboratory of one of us (L.W.E.) in Beltsville, Md. All the cultures have a history of original isolation from root nodules of leguminous plants.

Several types of media were tested for optimum motility and

flagellation. The best medium tried was either broth or agar of the following composition: peptone (tryptone or casitone) 0.5%, mannitol 1.0%, dipotassium phosphate 0.02%, magnesium sulphate 0.02%, calcium sulphate 0.01%, and sodium chloride 0.02%. Sometimes the best preparations were gotten from agar slants and sometimes from the liquid medium. Incubation was routinely at 20°C. for two or more days until distinct growth was obtained. With most strains the flagellation was rather poor, particularly with the peritrichously flagellated types. Only one culture appeared entirely atrichous.

The flagella stain of LEIFSON (1951) was used. The flagella of all strains stained readily. Measurements of flagellar wavelengths were made with a B & L filar micrometer according to the method of LEIFSON, CARHART and FULTON (1955).

The term *normal* as applied to a flagellar shape implies that this is the common or usual shape of the flagella of the particular organism in question. Distinctly different shapes are considered to be variants. Flagella of very short wavelength compared to the *normal*, usually from $1/2$ to $1/3$ the *normal* wavelength, are labeled *curly*. The other terms used in this paper for variant shapes are self explanatory.

EXPERIMENTAL RESULTS.

The type of flagellation, flagellar wavelengths and general growth rates are summarized in table 1. The photomicrographs in plates I, II, and III illustrate the flagellation of a typical organism from each of the plant species represented. Included are also illustrations of some of the observed flagellar variants.

Two major types of flagellation were observed in the *Rhizobium* strains studied: A peritrichous type and a subpolar monotrichous type. These two types differ both in the arrangement of the flagella and in the flagellar wavelength.

In the subpolar monotrichous type of flagellation the single flagellum originates close to the somatic pole with the proximal end of the flagellum typically at right angles to the long axis of the soma. This is distinctly different from the more usual polar monotrichous type of flagellation in which the proximal end of the flagellum is parallel to the long axis of the soma. Multiple subpolar flagella of *normal* wavelength at the same end were never observed, nor was a subpolar flagellum at each end ever observed. The flagellar

variants frequently showed several curly flagella at one pole. With most strains the subpolar flagella showed a uniform wavelength averaging from 1.9 to 2.2 microns. With some strains the subpolar flagellum was short with a single curve or hook, and on these satisfactory wavelength measurements could not be made. The organism illustrated in figure 11 shows quite clearly the origin of a subpolar flagellum from the surface of the soma. The subpolar monotrichous type of flagellation has never been observed by us in any genera of bacteria other than *Rhizobium*. Rhizobia with this type of flagellation may be identified by their morphology alone.

The strains with the peritrichous type of flagellation were generally poorly flagellated with most of the individuals atrichous. The most frequent number of flagella was one with relatively few organisms showing two or more flagella. In the single flagellated individuals the single flagellum, more often than not, originated at or near the somatic pole. In some strains individuals with two flagella also showed a strong polar tendency with both flagella originating at or near the somatic pole. This polar tendency is not unique with the peritrichously flagellated rhizobia but is common to many poorly flagellated peritrichous bacteria. This has frequently led to the mistaken labeling of such bacteria as polar flagellated. The wavelength of the peritrichous flagella was significantly shorter than that of the subpolar flagella, averaging from 1.3 to 1.6 microns. Peritrichous flagellation in rhizobia may thus be distinguished from subpolar by one of several characteristics: relatively short flagellar wavelength, lateral origin of flagellum, multiple flagella.

The rate of growth, judged by macroscopic observation of agar slant cultures, correlated almost perfectly with the type of flagellation. The strains with subpolar monotrichous flagella grew relatively slowly, and those with peritrichous flagella, relatively rapidly. With some exceptions, all the strains in a cross-inoculation group showed the same type of flagellation and consequently the same general growth rate. Most of the exceptions were in the cowpea cross-inoculation groups. Strains from the soybean and cowpea cross-inoculation groups were mainly subpolar, while those from alfalfa, clover, pea and bean (not lima) groups were peritrichous. Deviations from the normal or majority cannot be evaluated without additional information. Of 11 strains from *Phaseolus lunatus*, 9 showed subpolar flagellation and 2 showed peritrichous flagellation. There is nothing in the history of these cultures to indicate that the peri-

TABLE 1.
Flagellation and Growth Rates of *Rhizobium* Strains.

Cross-inoculation Group	Host Plant	Strain (ERDMAN)	Flagellation	Wavelength (μ)		Growth ¹⁾ Rate
				Normal	Variant	
Soybean	<i>Glycine hispida</i>	3I1b59	subpolar	2.05	0.80	++
	" "	3I1b66a	"	2.18	— ²⁾	++
	" "	3I1b76	"	1.95	—	+
	" "	" "	"	"	"	"
Cowpea	<i>Vigna sinensis</i>	3I6n10	"	2.00	—	+
	<i>Erythrina indica</i>	3I2b1	"	2.0-2.7	—	+
	<i>Phaseolus lunatus</i>	3I6d1	"	1.95	—	+
	" "	3I6d2	"	2.04	—	+
	" "	3I6d7	"	2.02	—	+
	" "	3I6d8	"	2.04	—	+
	" "	3I6d9	"	2.14	—	+
	" "	3I6d10	"	2.18	—	+
	" "	3I6d13	"	2.00	—	+
	" "	3I6d24	"	2.09	—	+
	" "	3I6d27	"	2.05	—	+
	" "	3I6d18	peritrichous	1.43	—	+++
	" "	3I6d24	"	1.56	—	+++
	<i>Phaseolus aureus</i>	3I6h1	subpolar	2.02	0.80	+
	" "	3I6h2	"	2.0-2.7	—	+
	" "	3I6h3	"	2.5	—	+
	" "	3I6h4	"	2.14	0.80	+
	" "	3I6h5	"	2.2	—	+
	" "	3I6h6	"	coiled	—	+
	" "	3I6h7	"	2.04	—	+
	<i>Phaseolus angularis</i>	3I6f1	subpolar	2.20	—	+
	<i>Phaseolus aconitifolius</i>	3I6g1	"	1.9	2.9	+
	" "	3I6g2	"	coiled	0.80	+
	<i>Phaseolus acutifolius</i>	3I6a1	"	coiled	—	+
	" "	3I6a3	"	2.05	—	+
	" "	3z6a4	"	2.05	—	+
	" "	3I6a5	"	2.08	—	+
	" "	3I6a6	"	2.24	—	+
	<i>Phaseolus species</i>	3I6k1	"	2.11	—	+
	<i>Ulex europaeus</i>	3C3a1	"	2.08	—	+
	<i>Pueraria thunbergiana</i>	3I4a8	"	2.14	0.75	++
	<i>Albizzia julibrissin</i>	1BOa2	"	2.00	—	+
	" "	1BOa1	peritrichous	1.0-1.6	—	+++
	<i>Acacia linifolia</i>	1AOc1	"	1.60	—	+++
Lupine	<i>Lupinus luteus</i>	3C2d1a	subpolar	coiled	0.75	+
	<i>Lupinus angustifolius</i>	3C2e3	"	coiled	—	+
	<i>Lupinus species</i>	3C2k5	"	2.05	—	+
	<i>Lupinus densiflorus</i>	3C2n1	peritrichous	1.52	—	+++
	<i>Strophostylus pauciflora</i>	3I6m1	peritrichous	1.45	—	+++
	" "	3I6m1a	"	1.48	—	+++
	" <i>helvola</i>	3I6l1	"	1.70	—	+++
	" "	3I6l2	"	1.42	—	+++
	" "	3I6l3	"	1.63	—	+++

TABLE 1 (continued).

Cross-inoculation Group	Host Plant	Strain (ERDMAN)	Flagellation	Wavelength (μ)		Growth ¹⁾ Rate
				Normal	Variant	
Alfalfa and Sweet Clover	<i>Medicago sativa</i>	3DOa30	peritrichous	1.60	—	+++
	" <i>hispida</i>	3DOd6	"	1.62	—	+++
	<i>Melilotus alba</i>	3DOh13	"	1.56	—	+++
True Clover	<i>Trifolium dubium</i>	3D1x3	"	1.25	—	+++
	" <i>repens</i>	3D1n4a	"	1.50	—	+++
	" <i>ambiguum</i>	3D1y8b	"	1.53	—	+++
Pea and Vetch	<i>Pisum arvense</i>			1.30	1.95	+++
	" "	3HOt21	"	1.30	—	+++
	" <i>sativum</i>	3HOq18	"	1.35	—	+++
Bean	<i>Phaseolus vulgaris</i>	3I6c1	"	1.55	—	+++
	" "	3I6c1a	"	1.30	—	+++
	" "	3I6c3	"	1.30	—	+++
	" "	3I6c3a	"	1.1-1.5	—	+++
	" "	3I6c6	"	1.50	—	+++
	" "	3I6c9	"	1.40	—	+++
	" "	3I6c10	"	1.35	—	+++
	" "	3I6c10a	"	1.37	—	+++
	" "	3I6c11	"	1.24	—	+++
	" "	3I6c12	"	1.37	—	+++
	" "	3I6c14	"	1.26	0.85	+++
	" " (navy)	3I6j2	"	1.30	—	+++
	<i>Lotus corniculatus</i>	3EOa8	"	1.54	—	+++
	" <i>americanus</i>	3EOb1	"	1.61	—	+++
	" <i>uliginosus</i>	3EOc7a	subpolar	2.18	0.75	+
	<i>Caragana arborescens</i>	3F6g1	peritrichous	1.50	—	+++
	" "	3F6g2	"	1.53	—	+++
	<i>Robinia pseudoacaceae</i>	3F4b3	peritrichous	1.54	—	+++
	" "	3F4b7	"	1.0-1.5	—	+
	<i>Amorpha fruticosa</i>	3F2d1	"	1.67	0.75	+++
	<i>Wisteria frutescens</i>	3F33c1	"	1.2-1.7	—	++
	" <i>speciosa</i>	3F3d1	subpolar	2.29	—	+

¹⁾ + means relatively slow growth, +++ means relatively fast growth on mannitol agar slants at 20° C.

²⁾ — means that variants were not observed.

trichous cultures are less authentic than the subpolar. Only by inoculation tests on lima beans can this be determined and such data are not available at present. The two strains studied from *Albizia julibrissin* differed morphologically and culturally. This plant species, and *Phaseolus lunatus*, were the only two species from which were obtained both flagellar types of rhizobia. However, in only a few instances were several strains from the same plant species included in the study. In several instances strains with each type

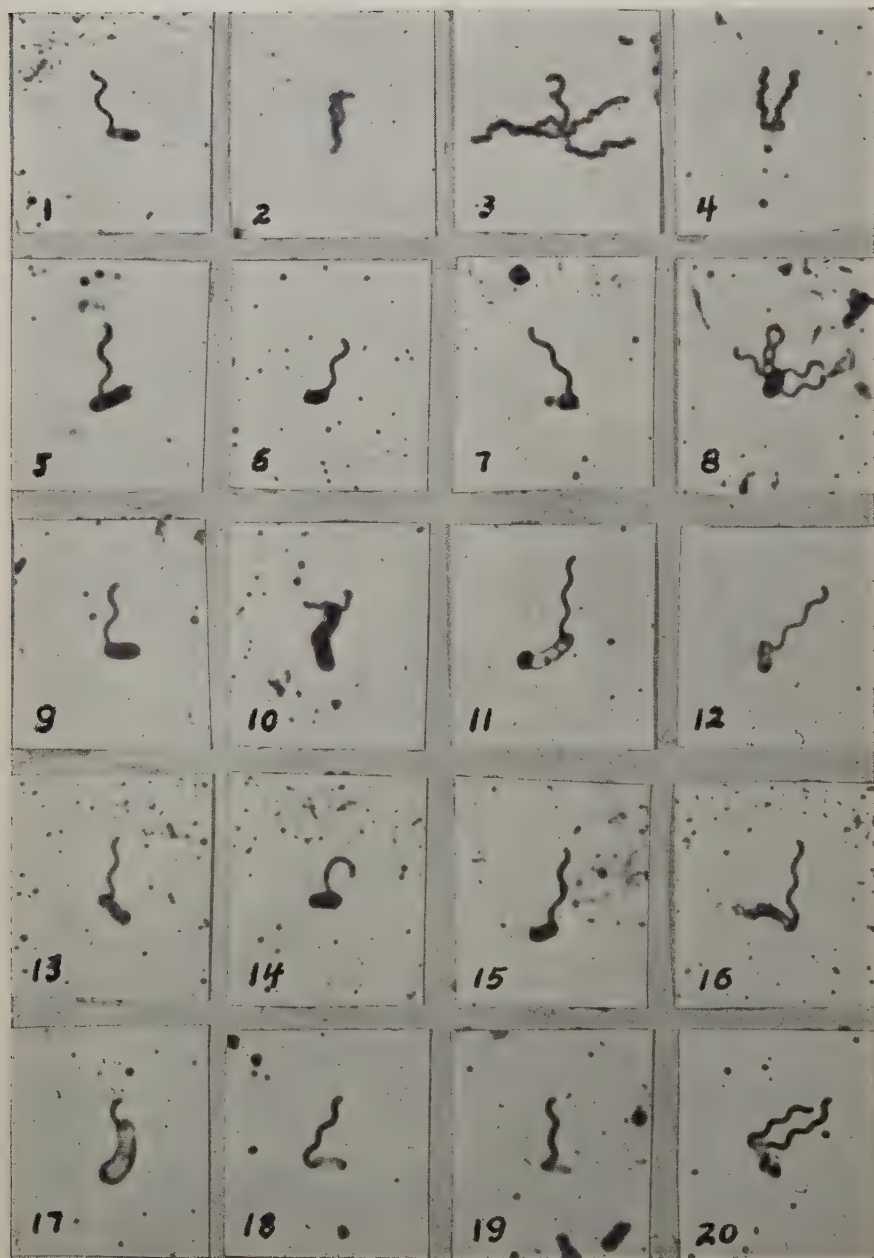


Plate I. Photomicrographs, magnification $2000\times$. LEIFSON flagella stain.

Figure 1, 2, 3, 4. From *Glycine hispida*, strain 3I1b59. Figure 1 shows the typical normal subpolar flagellum. Figure 2 shows the normal subpolar flagellum and one curly flagellum. Figure 3 shows four curly flagella in addition to the normal flagellum. The soma is rather indistinct. Figure 4 shows two curly flagella originating at the upper end of the lightly stained soma. Note the absence of the normal subpolar flagellum.

Figure 5. From *Glycine hispida*, strain 3I1b66a. A subpolar flagellum of normal wavelength but originating unusually far from the end of the soma.

Figure 6. From *Vigna sinensis*, strain 3I6n10. Normal subpolar flagellum.

Figure 7. From *Phaseolus lunatus*, 3I6d10. Typical normal subpolar flagellum. Most strains from *P. lunatus* were of this type.

Figure 8. From *Phaseolus lunatus*, strain 3I6d24. Peritrichous flagella of normal curvature. Two of the eleven strains from this plant species were of this type.

Figure 9, 10. From *Phaseolus aureus*, strain 3I6h4. Figure 9 shows the normal subpolar flagellum shown by all strains from this plant species.

Figure 10 shows a variant with two curly flagella.

Figure 11. From *Phaseolus aureus*, strain 3I6h7. Subpolar flagellum of normal curvature. Note the origin of the flagellum.

Figure 12. From *Phaseolus angularis*, strain 3I6f1. Normal subpolar flagellum.

Figure 13. From *Phaseolus aconitifolius*, strain 3I6g1. Normal subpolar flagellum.

Figure 14. From *Phaseolus aconitifolius*, strain 3I6g2. Subpolar flagellum with a single curve or hook. In some strains the majority of the flagella were of this type.

Figure 15. From *Phaseolus acutifolius*, strain 3I6a5. Normal subpolar flagellum.

Figure 16. From *Phaseolus acutifolius*, strain 3I6a6. This flagellum appears to originate from the very tip of the soma but otherwise it is a typical subpolar flagellum.

Figure 17. From *Phaseolus* sp., strain 3I6k1. The somatic origin of this short, hooked, subpolar flagellum is shown quite nicely.

Figure 18. From *Pueraria thunbergiana*, strain 3I4a8. Normal subpolar flagellum.

Figure 19. From *Albizia julibrissin*, strain 1BOa2. Normal subpolar flagellum.

Figure 20. From *Albizia julibrissin*, strain 1BOa1. Normal peritrichous flagella. Whether single or multiple, the flagella of this strain almost invariably originated at or close to the somatic pole.



Plate II. Photomicrographs, magnification 2000 \times . LEIFSON flagella stain.

Figure 21, 22, 23, 24. From *Erythrina indica*, strain 3I2b1. Figure 21 shows the normal subpolar flagellum. Most individuals in this strain had a short hooked subpolar flagellum. Figure 22 shows a typical hooked subpolar flagellum and two curly flagella. In figure 23 is shown a variant with three curly flagella in addition to the hooked subpolar flagellum. Figure 24 shows two curly flagella but the normal flagellum is absent.

Figure 25. From *Ulex europaeus*, strain 3C3a1. Normal subpolar flagellum.

Figure 26. From *Strophostylus pauciflora*, strain 3I6m1. Normal peritrichous flagella.

Figure 27. From *Strophostylus helvola*, strain 3I6I3. Peritrichous flagellation. In most strains of the peritrichous type a single flagellum per individual was very common.

Figure 28. From *Acacia linifolia*, strain 1AOc1. A rather unusually large number of peritrichous flagella.

Figure 29. From *Lupinus luteus*, strain 3C2d1a. A subpolar flagellum with rather uneven curvature.

Figure 30. From *Lupinus angustifolius*, strain 3C2e3. Subpolar flagellum.

Figure 31. From *Lupinus* sp., strain 3C2k5. Normal subpolar flagellum.

Figure 32. From *Lupinus densiflorus*, strain 3C2n1. Normal peritrichous flagella. This was the only peritrichous strain of the four tested from this species.

Figure 33. From *Medicago sativa*, strain 3DOa30. Normal peritrichous flagella.

Figure 34. From *Medicago hispida*, strain 3DOd6. This figure is included to show that a single peritrichous flagellum may have a polar origin. The strain is peritrichous flagellated.

Figure 35. From *Melilotus alba*, strain 3DOh13. Peritrichous flagellation. Flagella with a straight proximal part were also found in other strains of rhizobia.

Figure 36, 37. From *Trifolium dubium*, strain 3D1x3. Figure 36 shows the typical peritrichous flagellation of this species. Figure 37 probably represents two organisms with straight and partly straight flagella.

Figure 38. From *Trifolium ambiguum*, strain 3D1y8b. Peritrichous flagella of somewhat irregular curvature.

Figure 39, 40. From *Pisum arvense*, strain x. Peritrichous flagellation. The organism illustrated in figure 39 was not unusual for this strain.

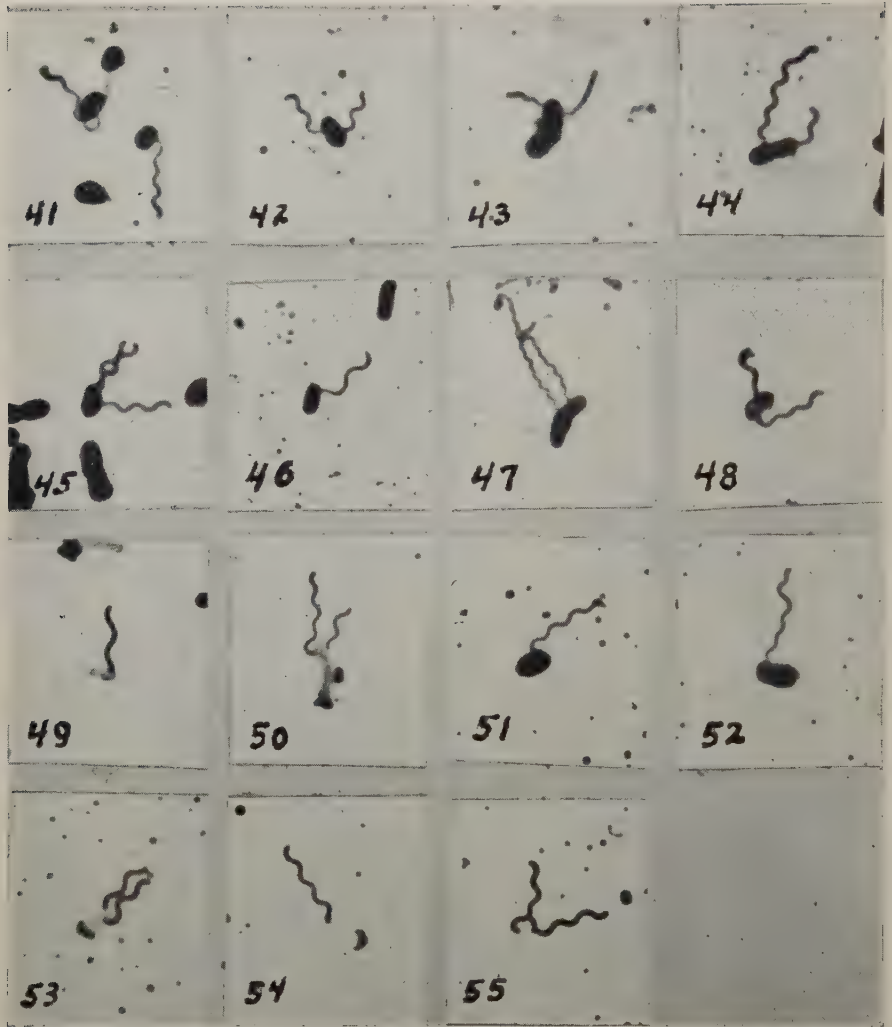


Plate III. Photomicrographs, magnification 2000 \times . LEIFSON flagella stain.

of flagellation were found in the same plant genus or the same cross-inoculation group. Strains with each type of flagellation were found in *Phaseolus*, *Albizzia*, *Lupinus* and *Wisteria*. Strains with each type of flagellation were also found in the cowpea and lupine cross-inoculation groups. All strains from a certain cross-inoculation group generally, however, showed the same type of flagellation.

Figure 41. From *Phaseolus vulgaris*, strain 3I6c3a. Normal peritrichous flagellation.

Figure 42. From *Phaseolus vulgaris*, strain 3I6c10a. Normal peritrichous flagellation.

Figure 43. From *Phaseolus vulgaris*, strain 3I6c14. A variant of the peritrichous type with one curly and one straight flagellum.

Figure 44. From *Lotus corniculatus*, strain 3EOa8. Normal peritrichous flagella.

Figure 45. From *Lotus americanus*, strain 3EOb1. Normal peritrichous flagella.

Figure 46, 47. From *Lotus uliginosus*, strain 3EOc7a. Figure 46 shows the normal subpolar flagellation characteristic of this strain. Figure 47 is a variant with curly flagella. The curly variants were frequent in this strain but usually the flagella were more polar in origin.

Figure 48. From *Caragana arborescens*, strain 3F6g2. Normal peritrichous flagellation.

Figure 49. From *Wisteria speciosa*, strain 3F3d1. Normal subpolar flagellum.

Figure 50. From *Wisteria frutescens*, strain 3F33c1. Multiple flagella of relatively short wavelength makes this a peritrichous type. In most individuals the flagella have a subpolar origin.

Figures 51, 52. From *Robinia pseudoacaceae*, strain 3F4b7. Figure 50 shows a normal peritrichous flagellum and figure 51 shows a variant with shorter wavelength flagella.

Figures 53, 54. From *Robinia pseudoacaceae*, strain 3F4b3. Peritrichous flagellation. Whether single or multiple, the flagella on most individuals were subpolar in origin.

Figure 55. From *Amorpha fruticosa*, strain 3F2d1. Normal peritrichous flagella.

FLAGELLAR VARIATIONS.

The peritrichous types showed only a few minor flagellar variations such as an occasional curly flagellum, a rare straight flagellum, and in a strain from *Pisum arvense* flagella of abnormally long wavelength (1.95) microns.

The subpolar types showed in several instances a most interesting flagellar variation observed in strains from *Glycine hispida*, *Erythrina indica*, *Phaseolus aureus*, *P. aconitifolium* and *Puereria thunbergiana*. In addition to the normal subpolar flagellum, single or multiple flagella of very short wavelength (about 0.8 microns) were observed. These curly flagella originated at or near the same somatic pole as the normal flagellum. In several instances were found individuals with only the curly flagella present. With one exception, the individuals with the curly or mixed flagella were relatively few. The one exception was the strain from *Lotus uliginosus*. On the slide made from the first transfer of this culture only individuals with curly flagella were observed. On one individual (see figure 47) the curly flagella were definitely lateral and the culture was classified as a curly variant of the peritrichous type. Since this culture was slow growing, like the subpolar types, several slides were made from fresh transfers. On these slides were found individuals with the normal subpolar flagellum as well as the curly variants. The original slide was then reexamined at great length and, at last, a single individual was found with the normal subpolar flagellum. Attempts at isolation of the curly variants in pure culture were not made and we have no information as to their stability.

DISCUSSION.

Judging from the data presented in this paper, strains of *Rhizobium* are of two major morphological types: 1) a fairly uniform rod-shaped organism with sub-polar monotrichous flagella of wavelength from 1.9 to 2.2 microns, and 2) a rather pleomorphic rod with peritrichous flagella of wavelength from 1.3 to 1.6 microns. The subpolar flagellated type grows relatively slowly in peptone-mannitol media, while the peritrichous type grows relatively rapidly. This correlation between morphology and growth rate is striking and almost perfect. Thus on the bases of morphology and general growth rate strains of *Rhizobium* may be separated into two fairly homogeneous groups. Each of these groups could be considered as constituting a species of *Rhizobium*. The genus *Rhizobium* would thus have only two species with possible further subdivision into varieties. However, we do not feel justified at present in making any specific taxonomic recommendations.

The flagellar variants, with multiple flagella of very short wave-

length, observed in the subpolar types, may indicate an evolutionary trend from subpolar flagellation to peritrichous flagellation. Here is a hint, perhaps, that the subpolar flagellated rhizobia have more recently developed phytopathogenicity, being evolved from an antecedent polar monotrichous type, compared to the peritrichous type of longer phytopathogenic history. From their close association with plants both types may eventually become entirely atrichous. The peritrichous types are not far from this condition at present.

Any indication of a life cycle in *Rhizobium* was not observed. The subpolar flagellated strains were uniformly small rods with little somatic variation. The peritrichous flagellated strains were somewhat more pleomorphic but large peritrichous bacteria which supposedly rupture to release smaller "swarmer" cells, as described by BISSET and HALE (1951) were not observed.

The statement by CONN and ELROD (1947) that strains of *Rhizobium* show both monotrichous and peritrichous individuals is misleading. These authors only studied strains from alfalfa, pea and clover which strains are peritrichous. As we have shown, these strains show many individuals with a single flagellum which may or may not have a polar origin. The term *monotrichous* should only be applied to cultures in which all flagellated individuals show predominantly single polar flagella. To avoid confusion the term *polar monotrichous* is preferable to *monotrichous*. The term *peritrichous* refers to a seemingly haphazard arrangement of the flagella which may or may not be multiple.

S u m m a r y.

The flagellation and growth characteristics of 82 strains of *Rhizobium* were studied. The strains were originally isolated from the root nodules of 19 genera and 35 species of leguminous plants.

Two morphological types of bacteria were found which differed mainly in the nature of their flagellation. The one type shows a most unusual and unique flagellation with single subpolar flagella of wavelength averaging from 1.9 to 2.2 microns. The other type shows peritrichous flagellation with usually one and, less often, several flagella per flagellated individual. The flagellar wavelength of the latter type averaged from 1.3 to 1.6 microns. Most strains of both types were rather poorly flagellated. An almost perfect correlation was found between the type of flagellation and the growth

rate in peptone-mannitol medium. The subpolar types grew relatively slowly and the peritrichous types relatively rapidly.

Some strains of the subpolar type showed flagellar variants with multiple flagella of very short wavelength in addition to the normal subpolar flagellum. A few individuals showed the short wavelength flagella only.

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Oslo, Norway).

TORULOPSIS NORWEGICA NOV. SPEC.

by

SIGNY REIERSÖL

(Received February 19, 1958).

This yeast, No 2166, was isolated from a sputum of a tuberculous patient.

Standard description of *Torulopsis norwegica*:

Growth in malt extract: After 3 days at 25°C. cells are round or short-oval $(2.4 - 5.2) \times (2.8 - 5.8) \mu$, single, in pairs and in small clumps. A thin ring and a sediment are formed. After one month at 17°C. a heavy ring and a sediment are formed.

Growth on malt agar: After 3 days at 25°C. cells are round or short-oval $(2.3 - 5.2) \times (2.5 - 6) \mu$. After one month at 17°C. the streak culture is cream-colored, glistening, almost smooth, margin almost smooth.

Slide cultures: No pseudomycelium is formed.

Fermentation: Absent.

Sugar assimilation:	Glucose	+	Maltose	—
	Galactose	—	Lactose	—
	Saccharose	—		

Assimilation of potassium nitrate: Positive.

Ethanol as sole source of carbon: Growth.

Splitting of arbutin: Positive.

Torulopsis norwegica nov. spec.

In musto maltato cellulae rotundae aut subovoideae $(2.4 - 5.2) \times (2.8 - 5.8) \mu$, singulae aut binae. Sedimentum et anulus formantur. In agaro maltato cellulae singulae aut binae $(2.3 - 5.2) \times (2.5 - 6) \mu$. Cultura (post unum mensem 17°C.) flavalbida, glabra, nitida, margine glabro. Pseudomycelium nullum. Fermentatio nulla. In medio minerali cum glucoso crescit. Nitratus caliculus assimilatur. In medio minerali cum alcohole etylico crescit. Arbutinum finditur.

DISCUSSION.

This yeast forms no pseudomycelium. Neither have we found ascospores. The cells are practically round with multilateral budding, and there is no production of a starch-like compound. It therefore has to be a *Torulopsis* species.

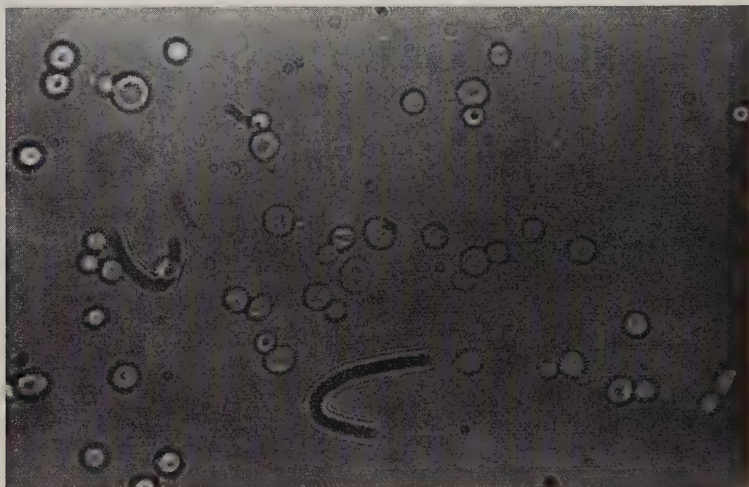


Fig. 1. *Torulopsis norvegica* 1000 \times .

Since we could not identify this yeast, No 2166, as a known species, it was sent to the Centraalbureau voor Schimmelcultures, Yeast Division, Delft. Dr SLOOFF who kindly examined the organism, also found that it could not belong to a known species, and she advised us to send a subculture to Dr WICKERHAM because he might possess mating types of the strain. WICKERHAM (1951) uses a great many carbon compounds in assimilation tests, and we are very much obliged to him for examining our strain with the compounds which he regularly employs. He found that the organism also assimilated cellobiose, rhamnose, glycerol, mannitol, salicin, pyruvate, lactate, succinate and citrate. His conclusion was that No 2166 did not key down to any of the known species on which they had data, but they had a few strains in an undescribed species which was fairly close to our species.

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(Laboratorium für Parasitologie der Reichsuniversität und Parasitologische Abteilung des Instituts für Tropenmedizin in Leiden).

DAS PROBLEM DER SPEZIFIZITÄT DES SABIN-FELDMAN FARBTESSTES ¹⁾

von

P. H. VAN THIEL

(Empfangen am 10. Januar 1958).

Im Unterstehenden möchte ich etwas mitteilen über die Untersuchungen der letzten Jahre meiner Assistenten VAN SOESTBERGEN und MAS BAKAL betreffs der Spezifizität des Sabin-Feldman Farbtestes (SFT), der in den Niederlanden neben der Komplement-bindungsreaktion bei der Diagnostik der Krankheit angewandt wird.

I. DIE TECHNIK DES SFT.

Der SFT gründet sich auf das nachfolgende Prinzip: Wenn Serum in dem sich Antikörper gegen Toxoplasma finden, zusammengebracht wird mit einer Suspension von Toxoplasmen, dann haben extrazellulär gelegene Parasiten in Anwesenheit einer konstanten Quantität Aktivatorserum nach einer Inkubationszeit von einer Stunde bei 37°C. ihre Affinität gegen Methylenblau verloren. Wenn es keine Antikörper im Serum gibt, wird der Farbstoff von der Mehrheit der Parasiten aufgenommen. Durch die Anfertigung einer Verdünnungsreihe des positiv reagierenden Serums kann man auf diese Weise ein Masz angeben für die Quantität der Antikörper in Serum. Dieser Titer des Serums ist die Endverdünnung des Serums welcher, in Anwesenheit einer konstanten Quantität "Aktivatorserums" noch imstande ist an 50 Prozent der Toxoplasmen ihre Affinität gegen Methylenblau bei pH 11 zu entnehmen.

Welchen Problemen begegnet man hier? 1. Das Problem der Spezifizität. Kann der Farbtest auch ein positives Resultat geben

¹⁾ Vortrag auf der I Tagung der tschechoslowakischen Parasitologen in Prag 6—10 Oktober 1957.

wenn es keine Toxoplasma-Antikörper gibt? 2. Im Zusammenhang mit der Frage unter 1: Deutet jeder Titer, auch die niedrigsten von 1 : 4, 1 : 8, 1 : 16, auf Toxoplasmosis?

In der Praxis der Diagnostik hat jedes Laboratorium einen unteren Grenztiter angenommen, z.B. VON ZEIPPEL und LINDER (1951) 1 : 10, WESTPHAL (1951) 1 : 25, SABIN u.M. (1952) 1 : 32, ROTH (1953) 1 : 64, VERLINDE (1954) 1 : 100. Eine Titersteigerung gibt entgültige Sicherheit über die Diagnose.

Die Frage ist unbeantwortet geblieben ob die genannten niedrigen Titer aspezifisch sind oder nicht. Im letzten Fall würde Toxoplasmosis bei der Bevölkerung von den Niederlanden und anderen Ländern sehr viel frequenter vorkommen als die serologisch deutlichen Fälle, die klinisch manifesten und asymptomatischen Fälle einbegriffen, angeben. Wahrscheinlich ebensowohl auf Grund der geringen Uebereinstimmung zwischen den verschiedenen Untersuchern über die geforderte Titerhöhe, sind die Angaben über die Frequenz der Toxoplasmosis bei der Bevölkerung sehr verschieden.

Niedrige Titer mögen also diagnostisch ohne Wert sein, epidemiologisch sind sie bedeutungsvoll. Bei der Untersuchung von 388 Militären (20–22 Jahre alt) beim Bluttransfusionsdienst fand VAN SOESTBERGEN in meinem Laboratorium einen Titer kleiner als 1 : 4 bei 36.9 Proz., Titer von 1 : 4, 1 : 20 und kleiner als 1 : 100 nacheinander bei 29.1, 17.0 und 11.6 Proz. (zusammen niedrige Titer bei 57.7 Proz.) und Titer von höher als 1 : 100 (1 : 256, 1 : 512 u. 1 : 2048) bei 5.4 Proz. Es macht epidemiologisch also Unterschied ob die Toxoplasmosis bei 5.4 Proz. oder bei 63.1 Proz. und mehr von jungen Männern vorkommt.

3. Die Reproduzierbarkeit der Reaktion. Im Laufe der letzten Jahre ist die Frage aufgekommen über die Reproduzierbarkeit der erzielten Titer. Ein selbes Serum zwei Mal nach der ursprünglichen Technik untersucht von VAN SOESTBERGEN zeigte einen Titer wechselnd zwischen drei Röhrchen, also z.B. 1 : 16 – 1 : 128, oder 1:128 – 1:1024. JIROVEC u.M. (1957) nennen schwankende Titerhöhen durch technische Fehler von 0 bis 1 : 500. Man hat angefangen zu suchen nach der Herkunft von störenden Einflüssen, die verschiedene Ergebnisse erklären könnten. WESTPHAL (1951) hält den Titer abhängig von der Marke vom Methylenblau. Dieser Auffassung wird aber von BEVERLEY und BEATTIE (1952), ROTH (1953) und von JACOBS und COOK (1954) widersprochen. JACOBS und COOK schreiben

die Unregelmäßigkeit einem hemmenden Faktor im Peritonealexsudat von mit Toxoplasma infizierten Mäusen zu. WILDFÜHR und HUDEMANN (1952) weisen auf die Verwendung von verschiedenen Parasitenstämmen hin, was von GARD u.M. (1949) bestritten worden ist. BEVERLEY und BEATTIE fanden einen direkten Zusammenhang zwischen dem Titer des Serums und der totalen Anzahl der im Peritonealexsudat vorhandenen Parasiten. Darum korrigierten sie die von ihnen gefundenen Titer. JACOBS und COOK konnten die Beobachtung von BEVERLEY und BEATTIE aber nicht bestätigen. BEVERLEY und BEATTIE sind auch der Meinung dasz die Quantität des Aktivators im Reaktionsmilieu von Einfluss ist. KÁSS (1954) will das Kriterium für den Titer von 50 Proz. ungefärbter Parasiten durch die Grenze zwischen 40 und 60 Proz. ungefärbter Parasiten ersetzen.

Fundamentaluntersuchungen waren also nötig um Licht auf diese Probleme zu werfen. VAN SOESTBERGEN (1956) hat die Grundlage für diese Untersuchungen in meinem Laboratorium gelegt.

Er ist ausgegangen von der Forderung der Reproduzierbarkeit der Reaktion in engen Grenzen. Zu diesem Zweck hat er die nachfolgende Technik entwickelt.

Technik des Sabin-Feldman Farbtestes (modifiziert von VAN SOESTBERGEN).

1. Inaktivierung des zu untersuchenden Serums.
2. Serumverdünnungen mit physiol. NaCl Lösung machen. Bei jeder Einpipettierung reine 1 cc Pipette (mit Chromsäure und gründlich mit Wasser gespült) benutzen.
3. In jedes Röhrchen 0.1 cc Serumverdünnung bringen.
4. Ansammlung von Toxoplasmen im Mäuseaszites am dritten Tag nach der i.p. Infektion der Mäuse.
5. Vermischung vom Aszites-Toxoplasmen-Gemisch 1 : 10 mit Aktivator (aus -30°C.), erwärmt bis 37°C. , in Fläschchen.
6. Schütteln vom Fläschchen (unter 5) mit der Hand während 7 Minuten = Aszites-Aktivatorgemisch.
7. 0.3 cc Aszites-Aktivatorgemisch (unter 6) mit 0.1 cc Serumverdünnung (unter 3) versetzen.
8. Jedes Röhrchen mit Gummistopf schlieszen und während 1 Stunde in Wasserbad von etwa 37°C. fortwährend schütteln.
9. In jedes Röhrchen 0.2 cc Methylenblau nach Sabin bringen und mischen.

10. Eine Nacht (eventuell länger) im Kühlschrank.
11. Röhrchen, bevor der Observierung von minimal 100 Parasiten per Röhrchen, mit der Hand gut schütteln.

Wenn Stichproben aus jedem einzelnen Röhrchen auf diese Weise genommen werden, sind die extrazellulär gelegenen Parasiten annähernd binomial verteilt. Der Antikörpertiter (der LD_{50}) ist auch hier diejenige Antikörperkonzentration die imstande ist 50 Proz. der extrazellulär gelegenen Parasiten ihre Affinität für Methylenblau zu nehmen.

Noch einige Bemerkungen werden jetzt gemacht:

1. Die Verdünnung des Serums wird notiert als die Endverdünnung des Serums wenn die Proberöhrchen im Wasserbad stehen (die Anfangsverdünnung wird also mit $\frac{1}{4}$ multipliziert); der später hinzugefügte Farbstoff wird also nicht mitgezählt.
2. Ein positives Kontrollserum musz stets mituntersucht werden. Dessen Titer bleibt, wenn das Serum eingefroren ist, sicher mehr als ein Jahr konstant.
3. Der Aktivator musz auf technisch richtige Weise gesammelt werden. Nötig ist frischer oder bei $-30^{\circ}C$. eingefrorener (nicht länger als ein halbes Jahr aufbewahrter) Aktivator mit einem hohen Gehalt an thermolabilen Substanzen. Wir fordern dasz nicht mehr als 15 Proz. der an dieses nicht-inaktivierte Serum hinzugefügten Parasiten (10 : 1), in Anwesenheit von Methylenblau, während einer Stunde bei $37^{\circ}C$. ungefärbt bleiben.
4. Hinzufügung des Farbstoffes. Es wurden zwei parallele Titrationen von einem positiven Serum gemacht und in der einen eine $1\frac{1}{2}$ mal gröszere Quantität der üblichen Quantität Methylenblau (Geigy) hinzugefügt. Dies hatte keinen Einfluss auf das Resultat der Reaktion. Das Pipettieren des Methylenblau braucht also nicht so genau stattzufinden als z.B. das Einpipettieren der Serumverdünnungen. VAN SOESTBERGEN hat nicht untersucht ob die Meinung von WESTPHAL betreffs des Einflusses der Marke des Methylenblau auf die Höhe der LD_{50} richtig ist. Vielleicht fungiert der Farbstoff nur als Indikator um eine schon eingetretene Zustandsänderung sichtbar zu machen.
5. Das Reaktionsmilieu verändert sich nicht nach Verbleib von 1-7 Tagen im Kühlschrank.

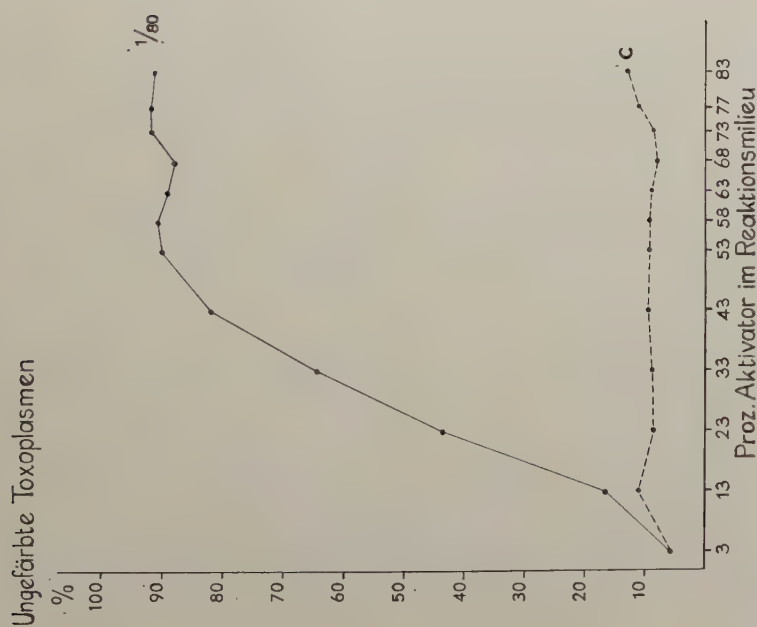


Fig. 1. Einfluss der Konzentration Aktivator auf den Prozentsatz ungefärbter Parasiten.

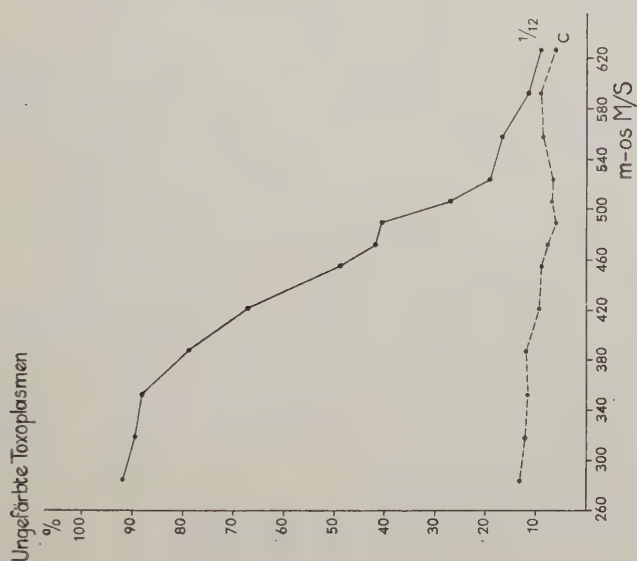


Fig. 2. Einfluss der Ionenkonzentration Na^+ und Cl^- auf den Prozentsatz ungefärbter Parasiten.

Näheres über die Reaktion.

1. Die Fig. 1 zeigt dasz der Aktivator bis einer Konzentration von ungefähr 50 Proz. einen wichtigen Einfluss auf den Prozentsatz ungefärbter Parasiten hat. Eine weitere Erhöhung dieser Konzentration hat keinen Einfluss. Umgekehrt ist es klar dasz man nicht erwarten kann dasz der maximale Effekt erreicht wird bei einer Aktivatorkonzentration von ungefähr 50 Proz. wenn die Aktivatorwirkung ungenügend ist.
2. Als Verdünnungsmedium wird physiologische Kochsalzlösung benutzt. Bei der Bestimmung der zweckmäßigen Konzentration zeigte es sich dasz der Prozentsatz ungefärbter Parasiten in derselben Menge positiven Serums kleiner wird je nachdem der osmotische Druck (in Milliosmol pro Liter) grösser wird. In diesem Zusammenhang weise ich auf die Notwendigkeit von gut gereinigtem Glasgeschirr, weil darin zurückgebliebene Salze die Reaktion ungünstig beeinflussen (Fig. 2).
3. Die Elektrolyte NaCl und inaktivierter Aktivator als Verdünnungsmedien benutzt, lassen denselben Prozentsatz Parasiten ungefärbt, in viel stärkerem Grad als die nicht-Elektrolyten Saccharose und Glucose (Fig. 3), wenigstens wenn die Konzentration dieser Verdünnungsmedia berechnet ist auf die milliosmoläre Konzentration von humanem Blut (300 m-osM/L). Wenn es Untersucher gibt die die unvorteilhafte Methode der Serumverdünnung mit Aktivatorserum benutzen, ist dies nicht fehlerhaft.
4. Die Reaktion, als chemischer Prozess betrachtet, ist nach sechs Stunden im Wasserbad noch nicht beendet (Fig. 4). Bei der Ausführung des Testes ist die Zeit von einer Stunde im Wasserbad gewählt.
5. Grosz ist der Einfluss der Temperatur des Wasserbades. Die Fig. 5 deutet darauf hin dasz man die Einpipettierungen ruhig bei Zimmertemperatur machen kann. Die Reaktion findet dann nur langsam statt.
6. Die Fig. 6 stellt die Sigmoidkurve dar des SFT eines positiven Serums, beruhend auf der Frequenzdistribution der Toleranzwerte von Parasiten Antikörpern gegenüber. Die p'' -Kurve gibt den Prozentsatz ungefärbter Parasiten an.

Wenn der Maximaleffekt der Reaktion D genannt wird, und der Minimaleffekt C, dann beteiligt 100-D Proz. Parasiten sich

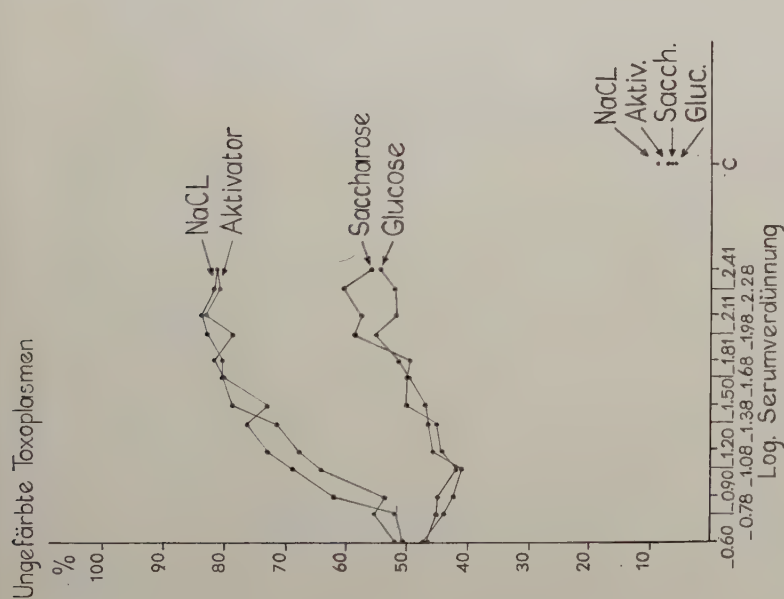


Fig. 3. Unterschied zwischen der Benutzung von Elektrolyten und nicht-Elektrolyten als Verdünnungsmedien.

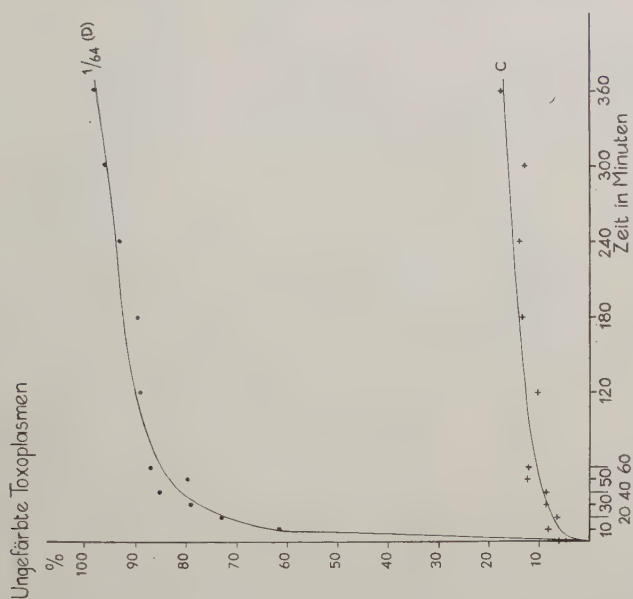


Fig. 4. Die Relation zwischen dem Prozentsatz ungefärbter Parasiten und der Zeit der Inkubation des Proberöhrchens im Wasserbad bei 38°C.

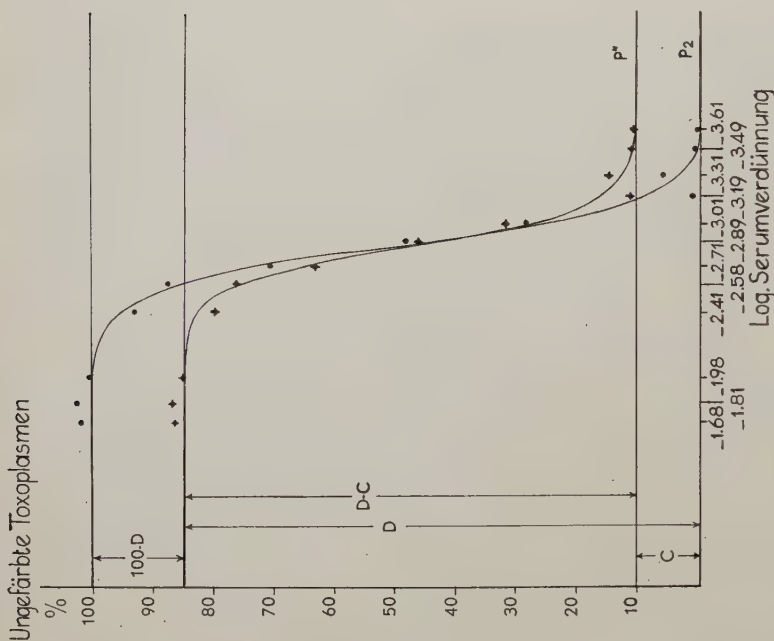


Fig. 6. Titration eines Serums mit wahrscheinlich Toxoplasma-Antikörpern. Vergleich der beobachteten (+ + +) und erwarteten Prozentsätze (P') ungefärbter Parasiten, mit den beobachteten Prozentsätzen (P₂) nach Transformation zu erwarteten Prozentsätzen P₁ und den erwarteten Prozentsätzen P₂.

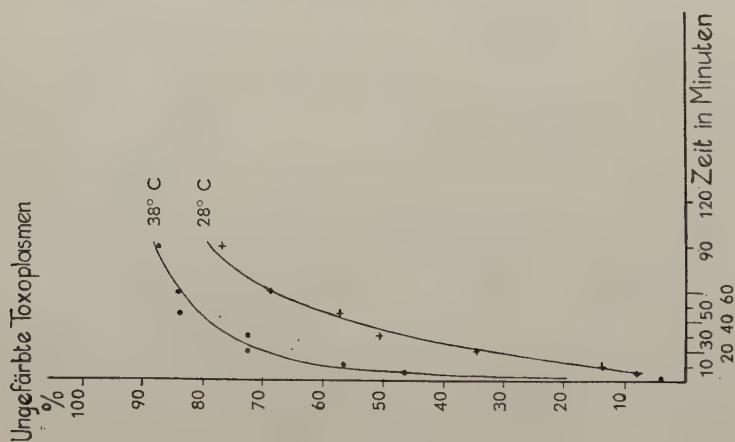


Fig. 5. Vergleichung des Belaufs der Reaktion bei 38°C. und bei 28°C. in einem Röhrchen in welchem das positiv reagierende Serum verdünnt ist bis 1/64.

nicht an der Reaktion, wahrscheinlich infolge des Schutzes den die Mäusewirtszellen den Toxoplasmen gegeben haben. Im Wasserbad und auch nach der Hinzufügung vom Farbstoff kommen nämlich noch Parasiten aus den Zellen frei wenn der Antikörper nicht mehr auf sie kann einwirken. Der Minimaleffekt C hat auch keinen konstanten Wert.

Die Gröszen 100-D und C beeinflussen das Resultat der Reaktion und die mögliche Genauigkeit womit dies erhalten werden kann ungünstig. VAN SOESTBERGEN hat jetzt die sehr wichtige Verbesserung angebracht die Grözse D-C auf 100 Proz. zu bringen dadurch dasz er die p'' -Werte umsetzte in p_1 -Werte, mit Hilfe der Formel:

$$p_1 = \frac{p'' - C}{D - C} \times 100\%.$$

Dadurch ist das Nachfolgende erreicht:

- a. Die Reaktion ist reproduzierbar geworden, d.h. dasz der Titer mit einem 95 Proz. Zuverlässigkeitsintervall gefunden wird das nicht grösser ist als 0.36 ($= \pm 2$ Mal die Standarddeviation).
- b. Die p_1 -Kurve (also die Toleranzverteilung der Parasiten in bezug auf spezifische Antikörper) entspricht annähernd der normalen Verteilung nach Gauss.
- c. Dadurch können Proberesultate nach statistischen Prinzipien, welche sich auf der normalen Verteilung gründen, bearbeitet werden.
- d. Sonst war die Quantität Antikörper nur charakterisiert durch den Log LD_{50} , = die Schätzung des Parameters μ . Jetzt ist ein zweites wichtiges Merkmal hinzugekommen, nämlich die Streuung σ der Parasitenpopulation in bezug auf die Antikörper. Ein aspezifischer Antikörper könnte einen selben Log LD_{50} , aber eine andere σ haben.
- e. Jetzt sind epidemiologische Untersuchungen mit Hilfe des SFT völlig zu vertreten.

Wenn nun die Relation zwischen Prozentsatz ungefärbter Parasiten und Logarithmus der Serumverdünnung bestimmt ist durch eine kumulative normale Verteilungskurve, dann müssen die in "Probits" transformierten Prozentsätze ungefärbter Parasiten um eine gerade Linie herum verteilt sein. Dies war tatsächlich der Fall. Die Sigmoidkurve wird dabei zu gerader Probit-Linie

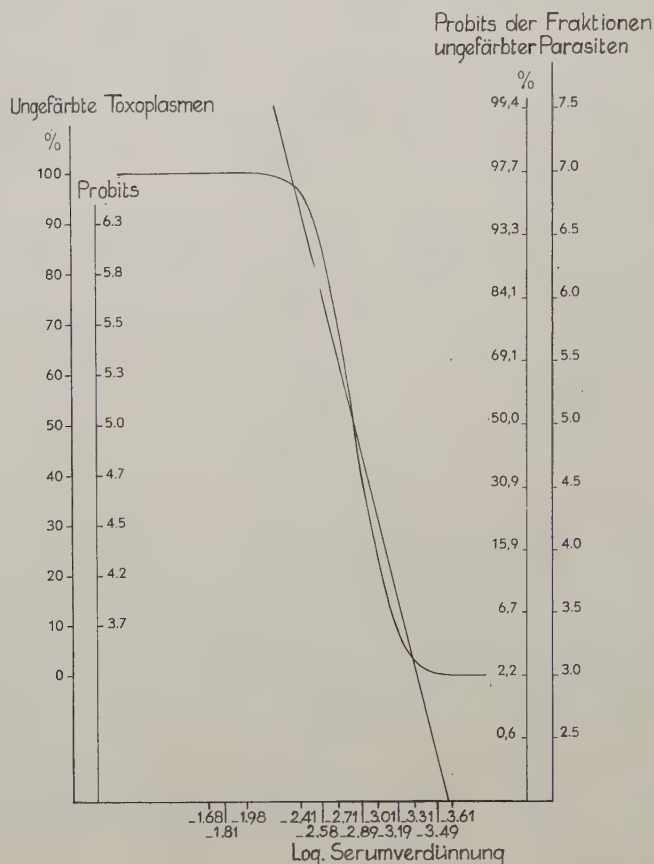


Fig. 7. In "Probits" transformierte Prozentsätze ungefärbter Parasiten einer kumulativen normalen Verteilungskurve.

(Fig. 7). Diese Linie wird bestimmt durch ihre Neigung β mit dem Absziss und durch die Schätzung von μ . VAN SOESTBERGEN hat also den Parameter β als typierende Konstante in den Resultaten von Serumtitrationen neben den Titer (LD_{50}) entdeckt. In wie weit der Wert dieses Parameters typisch ist für die Reaktion zwischen Toxoplasma und dem spezifischen Antikörper, muss näher untersucht worden. Durch die Bestimmung des Wertes von β ist prinzipiell die Möglichkeit gegeben aspezifisch reagierende Agentia von spezifischen Antikörpern zu unterscheiden. Dies braucht aber nicht der Fall zu sein.

Weiter hat VAN SOESTBERGEN gezeigt dass die geforderte Re-

produzierbarkeit von den Schätzungen der zwei Parametern μ und β , also von der Bestimmung von Log LD_{50} und b , völlig realisiert werden konnte. Die Schätzungen welche von den Parametern μ und β gemacht wurden, finden sich durchschnittlich in 95 Proz. der Fälle innerhalb eines Intervalls von ungefähr $x \pm 0.18$, beziehungsweise $b \pm 1.28$ (in logarithmischen Einheiten gemessen).

Ich erwähne noch kurz einige weitere wichtige Resultate aus der Arbeit VAN SOESTBERGEN'S.

1. All seine statistischen Untersuchungen wurden gemacht mit Serum 601 von einer klinisch gesunden Frau bei welcher von einem überstandenen Krankheitsprozesz nichts bekannt war. Der Titer ihres Serums betrug ungefähr 1 : 1024.

Die Möglichkeit bestand hier mit einer aspezifischen Reaktion zu tun zu haben. Um dieses Problem zu lösen wurden die Resultate der Titration von Serum 601 von VAN SOESTBERGEN (1956) verglichen mit der Titration des Serums eines Mannes, der sich mit Toxoplasma infiziert hat, aus dessen Lymphdrüsen der Parasit isoliert worden war und dessen Serum einen höchsten Titer von 1 : 1000 gezeigt hat.

Fig. 8 zeigt den Unterschied in b -Werten (auf dem Ordinaten finden sich die kumulativen Prozentsätze dieser Werte als Probits) mit den bezüglichen Zuverlässigkeitsintervallen. Der Unterschied zwischen beiden b -Werten (nacheinander 3.04 ± 0.48 und 4.2 ± 0.52) ist signifikant. Die Untersuchungen mit Serum 601 waren aber gemacht mit Toxoplasmen gezüchtet in "Champagne-Mäusen"; nach einer bakteriellen Infektion dieses Mäusestammes, war VAN SOESTBERGEN gezwungen das Serum des obengenannten Mannes mit Toxoplasmen zu untersuchen, die in einem weissen Mäusestamm gezüchtet waren.

Es stellt sich also die Frage ob dieser Unterschied in b verursacht wird durch die verschiedenen Mäusestämme oder durch die Aspezifität der Reaktion in Serum 601. Glücklicherweise verfügte VAN SOESTBERGEN noch über fünf Titrationen des Serums des Mannes mit Toxoplasmose bei denen Champagne-Mäuse für die Toxoplasmenzucht verwendet wurden. Alle fünf b -Werte fanden sich im Intervall 3.04 ± 0.48 , und ausser dem Intervall 4.2 ± 0.52 . Dies spricht dafür *a.* dasz Serum 601 tatsächlich Toxoplasma-Antikörper enthielt. *b.* dasz Mäusestämme Einfluss haben auf die Streuung der Toleranzdistribution

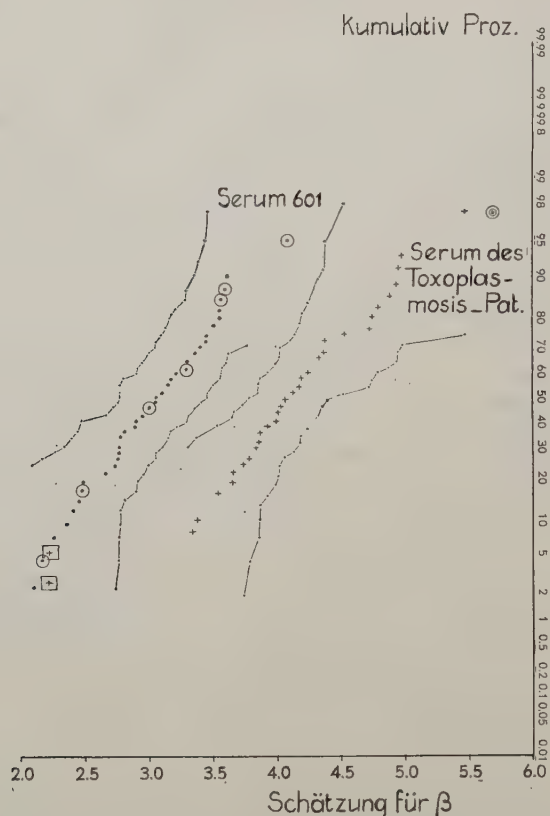
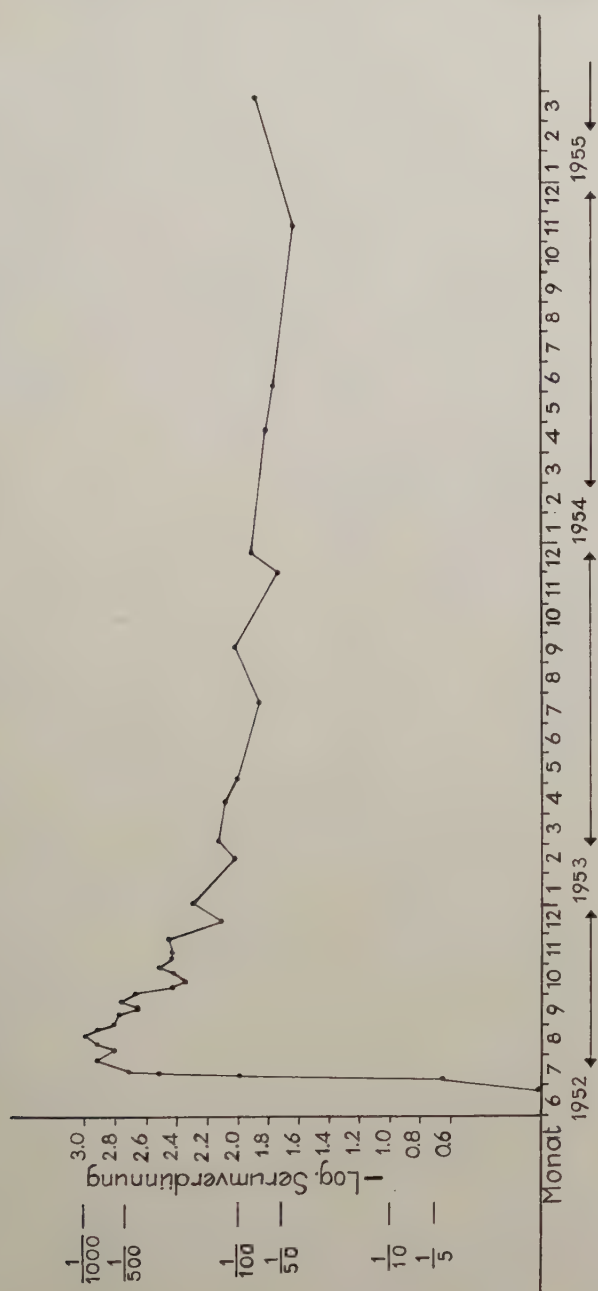


Fig. 8. Unterschied in b-Werten mit den bezüglichen Zuverlässigkeitsintervallen des Serums 601 und des Serums eines Mannes der sich mit Toxoplasma infiziert hat.

- bezüglich Toxoplasma-Antikörper. Es ist also nötig bei ähnlichen Untersuchungen den selben Mäusestamm zu verwenden.
2. Fig. 9 zeigt den SFT im Serum des unter 1 genannten mit Toxoplasma infizierten Mannes, ausgeführt nach der genauen Technik von VAN SOESTBERGEN. Das Interessante in dieser Infektion ist dasz der Mann sich während des Prozesses niemals krank gefühlt hat, obgleich er doch mit einem schwer virulenten Toxoplasma-Stamm infiziert war, denn 11 von den 12 mit Drüsenmaterial geimpften Mäuse sind an Toxoplasmosis gestorben (VAN SOESTBERGEN, 1957).
 3. Fig. 10 zeigt die kumulativen Prozentsätze in Probits der Schätzungen Log LD_{50} , ausgesetzt gegen den Wert dieser Schätzungen in Titrationen von Serum 601 mit vier Toxoplasma-



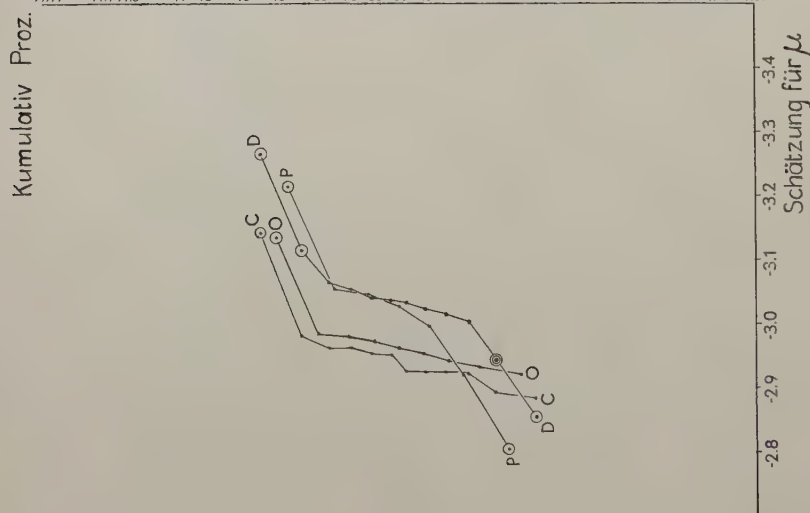


Fig. 10. Kumulative Prozentsätze in Probits der Schätzungen von Log LD₅₀, ausgesetzt gegen den Wert dieser Schätzungen in Titrationen von Serum 601 mit den Toxoplasma-Stämmen C, D, O und P.

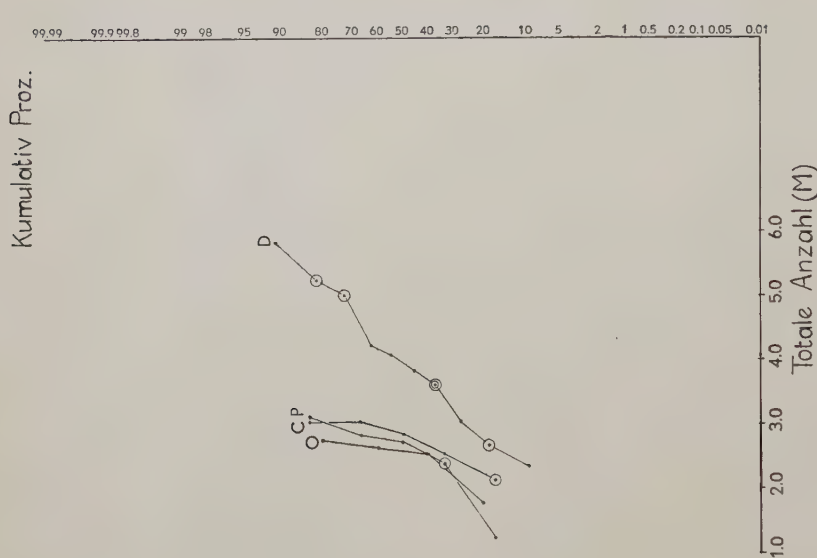


Fig. 11. Kumulative Prozentsätze in Probits der totalen Anzahl Toxoplasmen in Mäuseaszites, ausgesetzt gegen die Mittelwerte (in der Poisson-Verteilung) der Anzahl Parasiten in Aszites von Mäusen eingespritzt mit den Toxoplasma-Stämmen C, D, O und P.

stämmen. Dies suggeriert sofort dass diese Stämme tatsächlich verschieden sind.

4. Fig. 11 zeigt auf dem Absziss die M-Werte der Anzahl Parasiten im Mäuseaszites, d.h. den Mittelwert der für jede Zählung berechnete Poisson-Verteilung; auf dem Ordinat die Frequenzen als kumulative Prozentsätze in Probits. Die Beobachtungen mit dem Stamm Deelen zeigen sich am schönsten geradlinig verteilt (mit den anderen Stämmen sind wahrscheinlich zu wenige Versuche gemacht worden). Aber das wichtigste: beim Stamm Deelen hat der Aszites mehr Parasiten pro Volumeneinheit erhalten als der Aszites mit anderen Toxoplasma-Stämmen. Darum haben wir seitdem nur mit dem Stamm Deelen gearbeitet.
5. Ich habe schon gewiesen auf die Notwendigkeit den Aktivator auf richtige Weise zu sammeln, d.h. so schnell wie möglich einzufrieren und niemals anders als in frischem Zustand zu benutzen. Bei Benutzung eines Aktivators der ungefähr vor 6 Monaten oder früher bei einem Spender abgenommen war, oder bei Benutzung eines weniger wirksamen Aktivators, oder wenn der Aktivator kurz dauernd aufgetaut war, trat das Prozone-Phaenomen auf in den ersten Verdünnungsröhrchen, d.h. die Kurve der p'' -Werte nähert sich den Maximalwert in den niedrigsten Serumverdünnungen nicht asymptotisch, aber zeigte dort einen viel niedrigeren Prozentsatz ungefärbter Parasiten als auf dem Punkt der maximalen Reaktion (Fig. 12). Die reaktive Umsetzung der Parasiten hat dort also noch nicht genügend stattgefunden. Wenn die Wirksamkeit des Aktivators noch mehr zurückgegangen ist, äussert sich dies am ersten im nicht-Erreichen des Maximaleffektes in Verdünnungen mit Uebermasz Antikörper; weiter, bei noch weniger wirksamem Aktivator im Fallen des Maximaleffektes selber, bis schliesslich der hinzugefügte Aktivator keinen einzigen Einfluss auf den Lauf der Reaktion mehr ausübt. Das Auftreten des Zone-Phaenomens ist also abhängig vom Masz in welchem die reaktive Umsetzung in der gegebenen Zeitdauer stattgefunden hat. Dies ist wieder abhängig von der Quantität Aktivator, von dessen Wirksamkeit und von der Konzentration des Antikörpers. Bei niedrig positiven Titern wird die Prozone nicht beobachtet. In einer Kurve mit einer Prozone wie in Fig. 12 konnte VAN SOESTBERGEN doch Berechnungen machen, nämlich

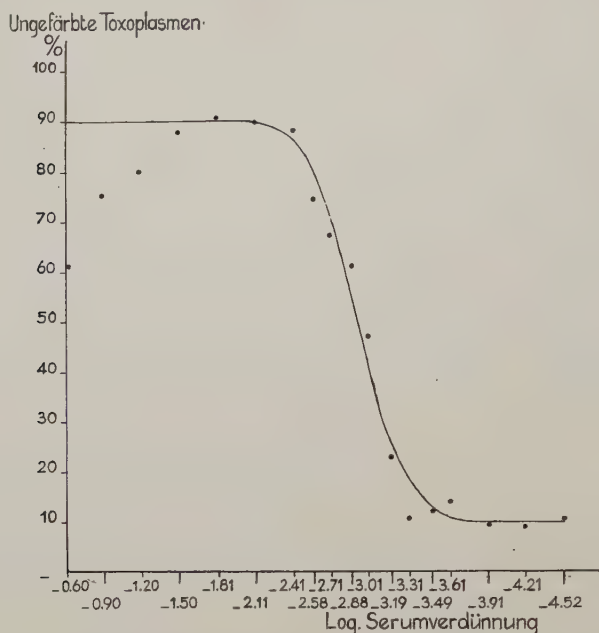


Fig. 12. Prozone in der Titration von Serum 601. Die beobachteten Prozentsätze ungefärbter Parasiten p'' und die Kurve der berechneten Prozentsätze P'' sind angegeben.

dadurch dasz er die p'' -Werte vor dem Maximum nicht mitzählte.

6. BEVERLEY und BEATTIE (1952) fanden eine direkte Beziehung zwischen der totalen Anzahl von im Peritonealexsudat anwesenden Parasiten und dem Titer des Serums. Dies hat VAN SOESTBERGEN nicht bestätigen können. Die Korrelationskoeffiziente berechnet zwischen der totalen Anzahl Parasiten Stamm Deelen in der Aszitesflüssigkeit, ausgedrückt als M, und den Schätzungen von den Parametern μ , β , D und C waren nicht signifikant abweichend von der Nullhypothese. Fig. 13 zeigt die Korrelationsdiagramme. Für weitere theoretischen Aspekten möchte ich weisen auf die Dissertation von VAN SOESTBERGEN.

II. UNTERSUCHUNGEN VON HUMANEN SEREN MIT NIEDRIGEN FARBTTEST-TITERN.

Frau MAS BAKAL ist jetzt beschäftigt mit der Anwendung der von VAN SOESTBERGEN gelegten Basis. Ich kann Ihnen jetzt mitteilen (die Resultate sind noch nicht publiziert und die Unter-

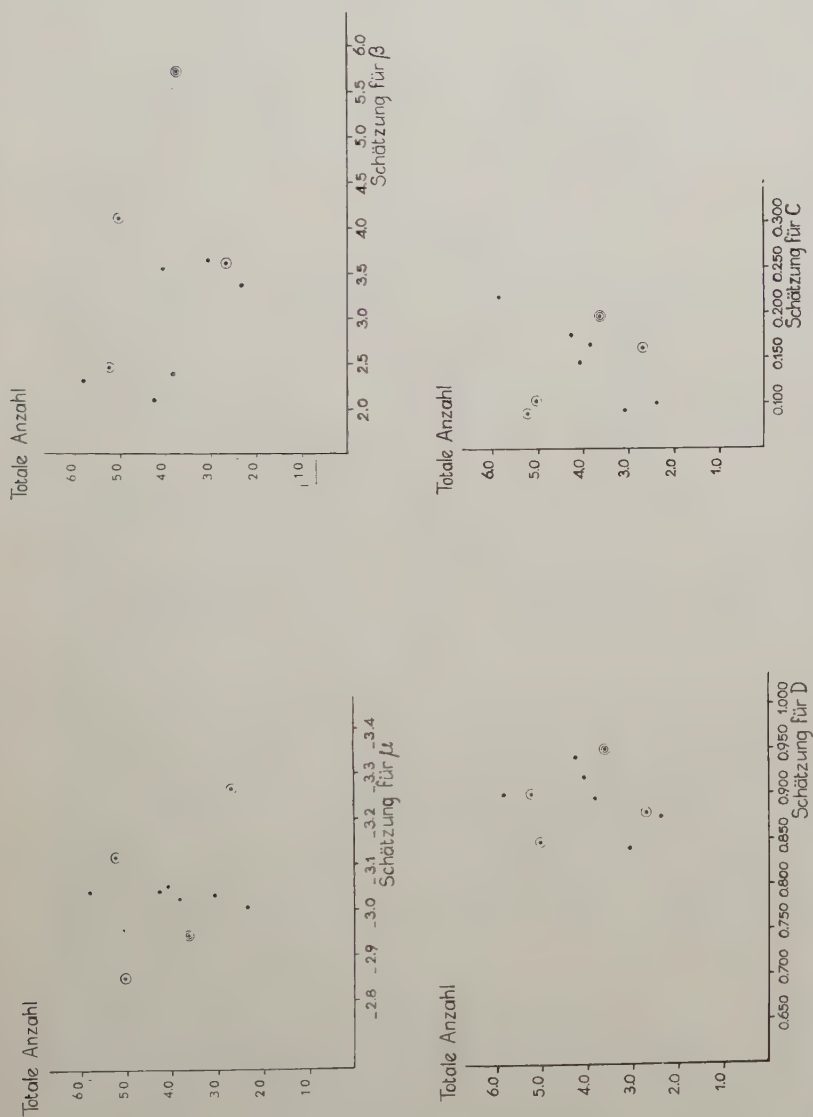


Fig. 13. Korrelation zwischen der totalen Anzahl Parasiten in der Aszites (ausgedrückt als Mittelwerte in der Poisson-Verteilung) und der Schätzungen von den Parametern μ , β , D und C.

suchungen nicht abgeschlossen) dasz die b-Werte von Titrationen von 19 humanen Sera mit Titern von 1 : 8 bis 1 : 256, berechnet nach der Probitanalyse, innerhalb der Zuverlässigkeitsintervallen der b-Werte im Serum des obengenannten mit Toxoplasma infizierten Mannes liegen, untersucht im SFT mit Toxoplasmen gezüchtet in weissen Mäusen und mit demselben Toxoplasma-Stamm Deelen. Dies heiszt dasz die niedrigen Titer in den untersuchten Sera durch Toxoplasma verursacht sein könnten.

III. VERSUCHE BEI KANINCHEN EINEN POSITIVEN SFT HERVORZURUFEN MIT ANDEREN KRANKHEITSERREGERN ALS TOXOPLASMA.

Hierzu hat Frau MAS BAKAL jedesmal drei Kaninchen eingespritzt mit Typhus-Paratyphus A. u. B.-Cholera Vakzin, Diphtherie-Toxin kombiniert mit Pertussis-Vakzin und mit den nachfolgenden lebenden Ansteckungsstoffen BCG-Vakzin, *Shigella sonnei*, Kuhpockenstoff, Influenza-Virus, Poliomyelitis-Virus und mit Cocksackie-Virus. Die vorher SFT negativen Sera dieser Kaninchen sind aber, soweit die Resultate jetzt vorliegen, negativ geblieben. Untersucht wurde nur nach der Methode von VAN SOESTBERGEN.

Eine gesonderte Besprechung fordern die Versuche die Frau MAS BAKAL gemacht hat mit Kaninchen und Mäusen, die infiziert wurden mit *Trypanosoma cruzi*, oder mit steigenden Dosen lebender Bakterienfreier *Trichomonas vaginalis* eingespritzt wurden, oder denen wiederholt oral *Sarcocystis tenella* vom Schaf und, in gesonderten Probeserien, oral *Sarcocystis hirsuta* vom Rind verabreicht wurde.

Diese gesonderte Erwähnung ist nötig, weil AWAD (1954a), AWAD und LAINSON (1954) die Spezifität des SFT in Abrede gestellt haben bei Anwesenheit von Antikörpern bezüglich *Trypanosoma cruzi* und *Trichomonas vaginalis*. Auch MICHALZIK (1953) vermutet dasz es eine Kreuzreaktion gibt zwischen Toxoplasma und *Trichomonas vaginalis* Infektion des Menschen. Bis jetzt hat Frau MAS BAKAL aber oben genannte Resultate nicht bestätigen können, obgleich *Trypanosoma cruzi* im peripheren Blut der Probetiere konstatiert wurde und obgleich eine Steigerung des Agglutinationstiters der postinfektiösen Sera der mit *Trichomonas* eingespritzten Kaninchen im Vergleich mit dem Titer der praeinfektiösen Sera stattgefunden hatte. Die Infizierung von Probetieren mit *Trypanosoma rhodesiense* konnte ebensowenig einen positiven SFT hervorrufen. Dieses Resultat mit *Trypanosoma cruzi* ist wichtig, weil

CATHIE (1957) in 15 Seren von Patienten mit der Krankheit von Chagas 6 SFT-positive mit niedrigen Titern gefunden hat.

Weiter haben MÜHLPFORDT (1951), AWAD (1954b), AWAD und LAINSON (1954) die Spezifität des SFT in Abrede gestellt kraft ihrer Fütterungsversuche von Kaninchen und Mäusen mit *Sarcocystis tenella*. In dieser Beziehung bemerkt GARNHAM (1957) dasz der Mensch, obgleich er nicht mit diesem Parasit infiziert wird, doch damit passiv immunisiert werden könnte, sodasz sich genügend Antikörper bilden könnten für einen positiven SFT. Bis jetzt ist es Frau MAS BAKAL nicht gelungen Mäuse mit *Sarcocystis tenella* oder *Sarcocystis hirsuta* zu infizieren durch Fütterung mit infiziertem Schaf- und Rindfleisch. Ueber das Resultat des SFT in diesen Mäuseren wird später berichtet. Unter Rindern in deren Herz *Sarcocystis hirsuta* gefunden wurde, fand sie sowohl Tiere deren Serum einen positiven SFT zeigte als Tiere bei denen dieser Test negativ war. AWAD und LAINSON (1954) und BEVERLEY (1957) beschrieben einen Patient mit Sarcosporidiosis, mit positivem Biopsiefund und mit negativem Farbttest.

Die Möglichkeit einer aspezifischen Reaktion in Seren von Menschen die mit *Sarcocystis* infiziertes Fleisch von Schlachtieren gegessen haben, ist also äusserst unwahrscheinlich. Ich achte sie aber ausgeschlossen auf Grund der nachfolgenden neulich von CATHIE (1957) angestellten Untersuchung. Bei der Untersuchung von 23 humanen Sera, welche nicht inaktiviert wurden, zeigte sich ein Drittel positiv im SFT mit Toxoplasma-Antigen, aber alle positiv mit *Sarcocystis*-Antigen. Nach Inaktivierung dieser selben Sera wurden sie aber alle negativ im SFT mit frischem *Sarcocystis*-Antigen; mit Toxoplasma-Antigen dagegen blieb die Reaktion beim genannten Drittel positiv. Mit Recht schloz CATHIE daraus dasz es keiner gemeinschaftlicher antigener Faktor in Toxoplasma und *Sarcocystis* gibt.

IV. DIE ASPEZIFISCHE WIRKUNG VON PROPERDIN.

PILLEMER u.M. (1954) isolierte "Properdin" aus Serum, ein Eiweisz, das in Verbindung stehen soll mit Komplement und das vielleicht etwas zu tun haben würde mit der bakteriziden Eigenschaft von Serum. GRÖNROOS (1955) hielt es für möglich dasz Properdin aus Serum im SFT nicht-spezifische Resultate geben könnte. Neben den Komplementfaktoren C_2 , C_3 und C_4 würde Properdin der wichtigste Stoff aus Aktivator sein.

Properdin wird bei Inaktivierung, $\frac{1}{2}$ Stunde bei 56°C., unwirksam gemacht (PILLEMER). Dies ist auch der Fall mit JETTMAR's "Toxoplasma feindlichem Faktor" (JETTMAR, 1954) und mit der "Wirksamkeit der Normalsera" von WESTPHAL und MÜHLPFORDT (1950). CATHIE (1957) hält beide erstgenannten Faktoren für identisch. Wie dem auch sei, Properdin aus Serum kann in unserer Ausführung des SFT niemals aspezifische Resultate geben, weil VAN SOESTBERGEN vorschreibt alle zu untersuchenden Sera zu inaktivieren.

Properdin aus Aktivatorserum kann auch keine Rolle spielen beim Entstehen einer aspezifischen Reaktion, auf Grund unserer Methode für das Suchen eines guten Aktivatorserums. Dies geschieht auf diese Weise: 0.5 cc nicht-inaktiviertes humanes Serum wird zusammen mit 0.05 cc Toxoplasma-Aszitesverdünnung während einer Stunde bei 37°C. gebracht. Properdin wird auf diese Weise nicht unwirksam gemacht. Das Serum darf erst dann als Aktivatorserum akzeptiert werden wenn nicht mehr als 15 Proz. Toxoplasmen ungefärbt bleiben. Mit anderen Worten: Als Aktivatorserum wird gerade ein Serum ausgesucht das so wenig wie möglich "Toxoplasma-feindlichen Faktor (Properdin oder JETTMAR-Faktor)" enthält.

Wenn ein exzeptionell hoher Gehalt an Properdin in Aktivatorserum anwesend sein würde, könnte man sich denken dasz eine aspezifische Reaktion entstehen würde. Dies kann aber niemals der Fall sein.

Ich mache darauf aufmerksam dasz GRÖNROOS selber (1955), ebenso wie später CATHIE (1957), geschrieben hat dasz das Serum in SFT inaktiviert werden musz und dasz er später (1956) in einer Diskussion es als seine letzte Erfahrung mitgeteilt hat dasz ein Versuch zu beweisen dasz das Properdin-System allein einen positiven Farbetest geben würde, mislungen ist.

Schlus sfolgerung.

Bis jetzt können keine Motive angeführt werden die gegen den Sabin-Feldman Farbttest als eine gegen Toxoplasma spezifische Reaktion sprechen. Nur die ganz genauen serologischen Untersuchungen nach VAN SOESTBERGEN sind imstande genau anzugeben in welchem Prozentsatz sehr niedrige Toxoplasma- Antistoff-Titer bei der Bevölkerung vorkommen.

Zusammenfassung.

Bei der Diagnostik der Toxoplasmosis, wie auch bei epidemiologischen Studien mit Hilfe dieses Testes, stößt man auf die Nicht-Reproduzierbarkeit in engen Grenzen und auf die Frage welcher Wert niedrigen Serumtitern beigelegt werden soll. Die Technik des Sabin-Feldman Farbtastes ist von VAN SOESTBERGEN studiert und verbessert worden, wodurch der Test auf diese Weise reproduzierbar geworden ist. Mit Hilfe dieser Technik und experimentell ist Frau MAS BAKAL beschäftigt Probleme der Aspezifität zu beantworten. Bis jetzt können keine Motive angeführt werden die gegen die Spezifität des Testes sprechen.

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STABILIZATION OF REDUCED STREPTOLYSIN-O FOR THE DETERMINATION OF ANTISTREPTOLYSIN IN BLOOD SERUM¹⁾

by

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In the Laboratory the antistreptolysin content of blood serum, which is considered to be of diagnostic value in acute rheumatic fever and other pathological conditions related to streptococcal infections, is determined by means of an inhibition reaction. The specific antigen is streptolysin-O in the form of a culture filtrate of a β -streptococcus. To us, strain S 84 is known to be a good streptolysin-producer. After incubation of the antigen with the serum under investigation and subsequent addition of rabbits' red blood cells the degree of hemolysis indicates the antistreptolysin content of the serum.

The most inconvenient feature of this procedure is, that streptolysin-O only exerts its action on a low oxydo-reduction level; it can be brought into a suitable state by reduction immediately before use. Cystein hydrochloride, sodium thioglycolate and sodium hydrosulphite ($\text{Na}_2\text{S}_2\text{O}_4$) have been used with almost equal success. The last one is the strongest, but at the same time the most unstable reducing substance; when a long series of titrations has to be done, even half an hour's delay is sufficient to cause a difference between the strength at the beginning and at the end of the series.

Re-oxydation by air-oxygen may be prevented by covering the reduced antigen with a layer of liquid paraffin; after some 24 hours an equilibrium is established at which a fairly good hemolytic activity exists which, when kept at -20 to -15°C ., is also fairly

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durable. However, as soon as the streptolysin is thawed up for titration, the unstability is the same as before. Besides, the paraffin oil sticking to the glass is an impediment for working to exact standards.

Kept at -20° in the oxydized state streptolysin-O remains unchanged for years. An equally stable product, immediately ready for use after thawing up, might be expected to result by adding an oxydo-reduction-stabilizer to the reduced antigen. As such hydroquinone was chosen.

The solvent or diluent used in the experiments is an isotonic veronal-buffer-solution of the following composition:

NaCl	8.4 g
Veronal	0.575 g
Veronal-Na	0.375 g
MgSO ₄ ·7H ₂ O	0.120 g
CaCl ₂	0.040 g

in 1000 ml distilled water; pH = 7.5

This solution may be stored in fivefold concentration.

The strenght of a streptolysin-O containing filtrate is expressed in minimum hemolytic doses (MHD) per ml. It is determined by adding 0.2 ml of a 5 % suspension of rabbit red blood cells in veronal buffer to each tube of a dilution series containing 1 ml of the streptolysin in dilutions $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, etc. The highest dilution which produces complete hemolysis after 30 minutes' incubation at 37°C . and subsequent cooling for 20 hours at 4°C . is the reciprocal value of the strength in MHD/ml.

A bottle of frozen, not reduced streptolysin-O was thawed up at room temperature and the contents were divided into 8 ml portions in 20 ml rubber-stoppered flasks. Sodium hydrosulphite was added to a final concentration of 100 mg/ml: in preliminary experiments this was found to be the most suitable concentration. Hydroquinone was added in different amounts as may be seen from table 1.

The flasks, marked \oplus , were kept at -20° , the others at 4°C .; at intervals specimina were taken for determination of the streptolysin activity. It should be kept in mind, that each titration means opening of the flask, letting in an amount of fresh air and, as far as they have been frozen, thawing up of the contents. The table shows, that when used immediately the higher concentrations of hydroquinone give the highest hemolytic activity. This may be due to the immediate stabilizing effect of the hydroquinone.

TABLE 1.

Portion nr	ml strep- tolysin	Na ₂ S ₂ O ₄		hydroquinone		veronal- buffer ml	Streptolysin MHD/ml after:				
		ml 1%	final conc. %	ml 2.5%	final conc. %		1 hour	weeks			
								1	2	3	15
1 ⊕	8	1	0.1	0.4	0.1	0.6	4	32	—		
3 ⊕	8	1	0.1	0.6	0.15	0.4	8	64	4		
5 ⊕	8	1	0.1	0.8	0.2	0.2	32	32	16	16	8
7 ⊕	8	1	0.1	1.0	0.25	0	32	32	32	16	16
2	8	1	0.1	0.4	0.1	0.6	8	32	—		
4	8	1	0.1	0.6	0.15	0.4	32	32	4		
6	8	1	0.1	0.8	0.2	0.2	32	32	8		
8	8	1	0.1	0.1	0.25	0	32	32	—		

⊕ kept at -20°C.

After a week reduction seems to be complete, whether stored at low temperature or not. When stored longer, the product seems the most stable at low temperature and after addition of the higher concentration of hydroquinone.

In control-tests without streptolysin it was demonstrated, that in the concentrations used none of the chemicals produces hemolysis by itself. In still higher concentrations, hydroquinone is less applicable because of its low solubility.

Having obtained the foregoing results, a stock of stabilized reduced streptolysin-O was made by adding 100 mg of sodium hydrosulphite and 250 mg of hydroquinone per 100 ml of culture filtrate. The product was divided into small volumes, covering about the need of a week and stored at -20°C. When not opened in between, the strength remained unchanged from 7 days after treatment up to 2 months. One batch remained constant at 32 MHD/ml, another at 64 MHD/ml.

S u m m a r y.

In the stabilization of streptolysin-O by adding 0.1 % of sodium hydrosulphite and 0.25 % of hydroquinone, followed by storing in deep-freeze in small quantities, we hope to have found a solution of one of the tricky problems of rheumatism serology.

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THE RELATIONSHIP BETWEEN *SACCHAROMYCES* *TELLUSTRIS* AND *CANDIDA BOVINA*

by

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INTRODUCTION.

In 1957 VAN DER WALT (1957a) described *Sacch. tellustris*. Strains of this species have been isolated from soil in South Africa, and from carious lesions in teeth of a Bantu (VAN DER WALT 1957b). According to VAN DER WALT's description the species is mainly characterized by round to short-oval cells, $(4-8) \times (4.3-9.5)\mu$; 1-2 round spores per ascus; fermentation and assimilation of dextrose only; no assimilation of nitrate. Further properties mentioned are the absence of pseudomycelium – only a few elongate cells may be formed –, assimilation of ethanol, and splitting of arbutin, the latter weakly.

Also in 1957 VAN UDEN and DO CARMO-SOUSA described a yeast isolated from the caecum of a cow, and named it *Candida bovina*. This yeast does not form spores, has oval to long-oval cells, $(4-5.9) \times (5.9-9.9)\mu$, and ferments and assimilates dextrose only. It forms pseudomycelium on corn meal agar. Nitrate and ethanol are not assimilated. The authors state that this yeast is rather fastidious in its growth requirements. They compare the species with the formerly described *Torulopsis pintolopesii* (VAN UDEN 1952), and consider them closely related. According to the authors the species differ in the size of the cells – *C. bovina* being bigger –, in the formation of pseudomycelium, and in the ability of *C. bovina* to grow at room temperature which *T. pintolopesii* fails to do. Perhaps there also exists a difference in habitat.

When reading the descriptions of *Sacch. tellustris* and *C. bovina* a relationship between them is not very obvious. In the first place

they were isolated from different sources. *C. bovina* is considered by the authors as a probable obligate saprophyte of warm-blooded animals. Stress is laid on the less vigorous growth of this species on culture media in comparison with other yeast species. This accent totally lacks in the description of *Sacch. tellustriis*.

EXPERIMENTAL.

Three of VAN DER WALT's strains of *Sacch. tellustriis* (CBS. 2685, 2686 and 2687) were studied by us. The results are in good agreement with the original description with a few slight exceptions: splitting of arbutin could not be confirmed, the assimilation of ethanol was very weak or negative. Sporulation appeared to be difficult. As indicated by VAN DER WALT spores could be observed on Gorodkova agar. A characteristic of the strains not mentioned in the description, is their relatively quick dying off. On malt agar they need to be transferred at least every two months, while most other yeast species survive a six months' subculturing.

The strain of *C. bovina* obtained from VAN UDEN (CBS. 2760) was studied at Delft and found to be in correspondence with the description. The formation of pseudomycelium was distinct, but primitive. Spores were not observed.

Later a second strain (CBS. 2761) of this species, isolated from the caecum of a horse, was obtained from VAN UDEN. Also this strain answered to the description.

When, at a given moment, struck by the similarity between *C. bovina* and *Sacch. tellustriis*, we retested the sporulation capacity of the *C. bovina* strains, spores were found in the second strain (CBS. 2761) on Gorodkova agar. This laid the bridge between *Sacch. tellustriis* and *C. bovina*, so that we may regard the latter as the imperfect stage of *Sacch. tellustriis*.

In strain CBS. 2761 single spores were isolated with the micro-manipulator. $\pm 80\%$ of the spores germinated and gave cultures, ten of which were used for study. The ten strains failed to form spores on Gorodkova- and V8 agar, though phenomena resembling conjugation between the cells were observed. Mating experiments did not yield sporulating cultures either¹⁾.

The strains did not all have the same vigour of growth, three

¹⁾ Dr J. LODDER was so kind as to prepare the one-spore cultures. Her sporulation and mating experiments led to the same negative results.

were markedly weaker. There were, however, no differences in temperature sensitivity. At 20°C. all grew.

In the course of the time several strains of the *T. pintolopesii* - *C. bovina* type had been received isolated from various sources. These strains, together with the two *C. bovina* strains mentioned before were used in a comparative study. They are listed below.

- CBS. 2760, *C. bovina*, received from VAN UDEN, isolated from the caecum of a cow.
- CBS. 2761, *C. bovina*, from VAN UDEN, isolated from the caecum of a horse.
- CBS. 1787, *T. pintolopesii*, type strain, received from VAN UDEN, isolated from white mice.
- CBS. 2676, from ORIE, isolated from mice.
- CBS. 2675, from PARLE, isolated from feces of rats and mice; indicated by PARLE (1957) as *T. pintolopesii*.
- CBS. 2678, from AUSTWICK, isolated from crop of turkey (AINSWORTH and AUSTWICK 1955).
- CBS. 2679, from AUSTWICK, isolated from lung of pullet.
- CBS. 2778, from VAN UDEN, originally from ASCHNER, isolated from caecum of a rat (ASCHNER *et al.* 1954); indicated by VAN UDEN and DO CARMO-SOUSA (1956) as *T. pintolopesii*.

In addition to strain CBS. 2761 spores were found in the strains 2676, 2675 and 2679 (cf. Table 1). In all instances one, seldom two spores per ascus were present. The sporulation medium of choice was Gorodkowa agar.

Morphologically the differences between the eight strains studied appeared to be slight. Between the strain of *T. pintolopesii* (CBS. 1787) and all the other strains no distinction could be made in the shape and the size of the cells in malt extract and on malt agar. The medium size in malt extract found was $(4.5-9) \times (6-10) \mu$. One to two times subculturing with two to three days in between clearly favoured the vigour of growth of the cells. The incubation temperature chosen was 30°C. At 37°C. all strains grew well, but a drawback was that after 2-3 days at this temperature, a large number of cells appeared to be in a bad condition. The cells, showing a granular appearance were markedly smaller than the healthy looking ones. The malt agar cultures of *T. pintolopesii* (CBS. 1787) and CBS. 2676 after 3 days at 37°C. were brown, of the other strains yellowish-brown.

The formation of pseudomycelium varied for the different strains. The development was best on corn meal agar. If present, it was

primitive as in the type strain of *C. bovina*. In several cases only elongate cells appeared. In *T. pintolopesii* even these were not observed.

Of the physiological properties, two, not commonly used in the differentiation of yeast species, were taken into consideration, since stress was laid on them by VAN UDEN and DO CARMO-SOUSA. They are the temperature of growth and vitamin requirements.

Two of the strains, *T. pintolopesii* (CBS. 1787) and CBS. 2676 did not grow at 20°C. All the other strains, *Sacch. tellustriis* included, grew, if slowly. At 25°C. all strains grew on malt agar, although the first two mentioned grew very slowly. At 30°C. there was fair growth by all strains.

The vitamin requirements of the strains were tested according to the method devised by VAN UDEN and DO CARMO-SOUSA (1956) for these species. In view of the latter authors' findings the vitamins used in the experiments were restricted to six: thiamine hydrochloride (400 µg/l), pyridoxine hydrochloride (400 µg/l), niacin (400 µg/l), calcium pantothenate (400 µg/l), biotin (2 µg/l), and inositol (2000 µg/l). As a basic medium served the vitamin-free liquid medium of WICKERHAM (1951). A tube containing this medium only served as a negative control, one with all the vitamins added as a positive control. To the other tubes five of the vitamins, omitting one in turn, were added. The temperature of growth was 30°C., in contrast to 37°C. chosen by VAN UDEN and DO CARMO-SOUSA, because the cells were found to die off quickly at 37°C. As inoculum was used a suspension of a fresh culture on malt agar in the basic medium kept for 24 h at 30°C. for exhaustion. The tests were read after one week.

Besides the 8 strains in study, three strains of *Sacch. tellustriis*, two single-spore cultures of CBS. 2761 (2761-1 and 2761-2), and two strains of *C. slooffii*, a related species, were taken into consideration. The results are listed in Table 1.

The table shows that all strains tested need niacin for growth. All can grow without inositol, with the exception of the two *C. slooffii* strains. For the other four vitamins tested it is impossible to distinguish between groups. *T. pintolopesii* (1787) is, besides *C. slooffii*, conspicuous for its great vitamin requirement, but also the strains *Sacch. tellustriis* (2687), CBS. 2761-1, and 2778 are notable for that. The strain *Sacch. tellustriis* 2687 is in its growth on malt agar clearly less vigorous than the other two strains of this

TABLE 1.

Vitamin requirements and sporulation.

Name or origin of the strain	CBS. No	niacin	inositol	thiamine	pantothenate	pyridoxine	biotin	Sporulation
<i>Sacch. tellustriis</i>	2685	—	+	+	±(w)	+	—	+
<i>Sacch. tellustriis</i>	2686	—	+	+	+(w)	+(w)	+(w)	+
<i>Sacch. tellustriis</i>	2687	—	+	±(w)	±(w)	—	—	+
<i>C. bovina</i>	2760	—	+	±(w)	+	+	—	—
<i>C. bovina</i>	2761	—	+	+	+	+	+	+
one-spore culture	2761-1	—	+	—	+(w)	—	—	—
one-spore culture	2761-2	—	+	+	+	+(w)	—	—
<i>T. pintolopesii</i>	1787	—	+	—	—	—	—	—
strain from ORIE	2676	—	+	±(w)	+	—	—	+
strain from PARLE	2675	—	+	—	±(w)	+	±(w)	+
strain from AUSTWICK	2679	—	+	±(w)	+	—	—	+
strain from AUSTWICK	2678	—	+	+	+	+	+(w)	—
strain from ASCHNER	2778	—	+	—	±(w)	±(w)	±(w)	—
<i>C. slooffii</i>	2419	—	—	—	+	—	—	—
<i>C. slooffii</i>	2783	—	—	—	+	—	—	—

Legend: The name of the vitamin in the head of the column indicates that in the experiment in question this was the vitamin lacking in the medium. In these columns + = growth; — = no growth; ± = in some experiments growth, in others not; (w) = weak growth.

species. The difference between two single-spore cultures from the same strain, 2761-1 and 2761-2, is noteworthy; 2761-1 belongs to the less vigorous type. VAN UDEN and DO CARMO-SOUSA (1956) found that *C. slooffii* also needs pantothenate for growth. We found the same at 37°C.; but at 30°C. it did not require it.

DISCUSSION.

From the study of the original strains of *Sacch. tellustris*, and the strains indicated as *C. bovina* or *T. pintolopesii*, or as yet unnamed in which spores have been found we may conclude that VAN DER WALT's description of *Sacch. tellustris* ought to be completed with a few characteristics. First of all the short-livedness on malt agar of the strains should be mentioned. Also the possibility of formation of a primitive pseudomycelium may be left open. The strains found to belong to this species are: CBS. 2761 (from VAN UDEN), CBS. 2675 (from PARLE) and CBS. 2679 (from AUSTWICK). For strain CBS. 2676 (from ORIE) we would like to make a restriction. The non-sporogenous forms (CBS. 2760, 2678 and 2778) can be retained in *C. bovina*.

VAN UDEN and DO CARMO-SOUSA (1956) indicate as the *glabrata*-group the species *T. glabrata*, *T. pintolopesii*, *C. bovina* and *C. slooffii*. The authors point to the close relationship between these species which all ferment and assimilate dextrose only, and possibly are all saprophytes of warm-blooded animals.

In our opinion *T. glabrata* takes a place apart in this group. It is easily distinguishable from the other species. The cells are smaller, on average $(2.5-5) \times (3.5-6)\mu$, while the cells of the other are on average $(4.5-9) \times (6-10)\mu$. Further *T. glabrata* is not short-lived.

In the differentiation of the other three members of the group we have only a few tools. There is first of all the formation of pseudomycelium. In the strains of *C. slooffii* studied the development was generally pronounced and gave no rise to doubt. In the other strains it varied from none at all to longish cells and primitive shapes. Secondly a differentiation based on the requirement of various vitamins is very difficult as appears from table 1. Also in this instance perhaps *C. slooffii* makes an exception being the only species needing inositol for growth. As a last resort growth at 20°C. could be considered for differentiation. *T. pintolopesii* and *C. slooffii* do not grow at this temperature.

From this appears that *C. slooffii* is recognizable using these three

characteristics. *T. pintolopesii* distinguishes itself from *C. bovina* by the growth temperature.

This brings us to the question which value should be attached to these properties for the differentiation in species in general and in this special case.

VAN UDEN and DO CARMO-SOUSA point to the fact that the use of the formation of pseudomycelium as a means to distinguish between genera may lead to artificiality, in this instance by separating *T. pintolopesii* and *C. bovina* and *C. slooffii* in different genera. The formation in these species has by its primitive shape a limited value.

The temperature of growth and the vitamin requirements have not yet found common use in the differentiation of species, but rather in the characterization of strains. There were found to exist differences in vitamin requirements in strains of the same species, *i.e.* in *Sacch. cerevisiae*. It is also well known that the vitamin tests should be strictly standardized.

From the vitamin tests recorded above appears that, first of all the method used gave variable results for a number of vitamins, and secondly that single-spore cultures from the same strain differed in requirements. The latter makes these vitamins for the differentiation of species in this case irrelevant. This may, however, not hold for all vitamins.

The use of the temperature of growth leads here to the separation of *Torulopsis pintolopesii* and *Candida bovina*. Whether this use is justified, is still questionable. Nevertheless we would prefer to retain these species separate, if provisory.

This problem also arises for the sporulating strain CBS. 2676 which, in contrast to *Sacch. tellustris*, does not grow at 20°C. Has it to be regarded as a sporulating *T. pintolopesii* or as a thermophilic *Sacch. tellustris*? The isolation and study of more strains not growing at 20°C., either sporulating or not, can perhaps aid us in the solution of the problem.

S u m m a r y.

A short survey of the descriptions of *Sacch. tellustris* and *C. bovina* is given with commentary induced by the results of our experiments. From this appears the relationship between the species, namely *Sacch. tellustris* being the perfect stage of *C. bovina*.

The results of a comparative study of the strains of *Sacch. tel-lustris* and *C. bovina*, and of the strains of related species is given. This study comprises some morphological features, and of the physiology, temperature relations and vitamin requirements.

In the discussion the taxonomic position of the related species is considered.

A c k n o w l e d g e m e n t .

I am much indebted to Dr J. LODDER for helpful advice in the composition of this paper. My thanks are due to Miss H. I. CUNO for technical assistance.

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SOME OBSERVATIONS ABOUT THE CAMP REACTION AND ITS APPLICATION TO HUMAN β HAEMOLYTIC STREPTOCOCCI ¹⁾

by

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In 1944 CHRISTIE, ATKINS and MUNCH-PETERSEN described a haemolytic phenomenon caused by group B streptococci (LANCEFIELD's groups) on sheep blood agar plates with colonies of β toxin producing staphylococci surrounded by darkened zones. Colonies of group B streptococci growing on or near these zones give rise to wide areas of complete haemolysis, but only within the borders of the β toxin zones. Such β toxin producing staphylococci are most commonly found in animals (MINETT 1936, COWAN 1938) and less frequently in men. Studies of β toxin were made by BRYCE and ROUNTREE (1936), CHRISTIE and NORTH (1941), VAN HEYNINGEN (1950) and others.

CHRISTIE and GRAYDON (1941) pointed out that certain non-haemolytic staphylococci in a zone of β toxin also produce haemolysis, which is probably caused by staphylococcal lipase.

It could be ruled out that the responsible agent of the group B streptococci is a lipase, a fibrinolysin or a casein digesting enzyme. The extracellular, filtrable and thermostable agent alone does not cause visible alterations of sheep red cells. However in combination with β toxin from staphylococci the agent brings about complete haemolysis of sheep and ox red cells. No lysis can be obtained with horse, human, rabbit and guinea-pig blood.

The lytic phenomenon, called CAMP reaction (initials of first authors)

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on proposal of MURPHY *et al.* (1952), is now widely used in the veterinarian bacteriology for the detection of group B streptococci (*Str. agalactiae*) in bovine mastitis. The material is normally brought on the primary blood plates, which then are inoculated in various ways as spots or streaks with a β toxin producing *Staphylococcus* strain. The reaction can be performed also with isolated pure strains. MUNCH-PETERSEN and CHRISTIE (1948) drew already attention to the characteristic shapes of the haemolytic areas in this reaction. For instance, if a colony of β toxin producing staphylococci and a colony of group B streptococci are growing about 1 till 1.5 cm apart on a sheep blood plate, around the staphylococcal colony a round darkened zone develops with a peculiar shaped area of complete haemolysis between the two colonies. The shape varies from that of a concavo-convex lens to a segment according to proximity of colonies, instead of the shape of a biconvex lens that could be expected as a result of the overlapping of two round diffusion zones. Haemolysis in the β toxin zone however is less extended than corresponds with an undisturbed diffusion of the streptococcal agent. Authors suggested three possible explanations:

1. The streptococcal agent is being used up in the β toxin zone as it advances through the darkened zone, perhaps by being adsorbed.
2. The red cells (or medium) in the darkened zone resist diffusion.
3. A combination of both.

Positive CAMP reactions are characteristic for group B streptococci. CHRISTIE *et al.* (1944) found all 64 examined strains positive. MUNCH-PETERSEN *et al.* (1945) reported 200 group B strains (120 bovine and 80 human strains) to react positive without exceptions: 395 other streptococcal strains (213 A, 93 C, 2 D, 2 E, 64 G, 1 H, and 20 ungrouped) did not produce the lytic agent or did so to a negligible degree. BARNUM (1950) saw amongst 275 bovine group B streptococci 98.2% with a positive CAMP reaction. MURPHY *et al.* (1952) examined 322 group B strains of which 96.6% reacted positive. Of 261 cultures classed as *Str. uberis* 39 or 14.9% produced some degree of reaction. All 46 strains of *Str. dysgalactiae* were negative. Serological grouping was not performed on all strains. In a study of FEY (1953) 99% of 200 bovine group B streptococcal strains were CAMP positive and none of 27 strains of *Str. dysgalactiae* (group C): only 2% of 157 other streptococcal strains not belonging to the groups B and C (e.g. *Str. uberis* and Enterococci) gave positive CAMP reactions.

The present study was made to gather some further information about the mechanism of the CAMP reaction and to test this reaction on β -haemolytic streptococci from human sources.

BACTERIOLOGICAL PART.

Methods and material.

1. **Blood.** For the CAMP reactions 5% sheep blood agar plates were used. In our experience the reactions with sheep blood are generally larger and more distinct than with ox blood. Probably because the defibrinated blood as a rule came from young sheep (through the intermediary of the veterinarian service of the slaughterhouse) we seldom met blood samples unsuitable for the purpose. The sheep serum namely may contain staphylococcal β antitoxin (MUNCH-PETERSEN *et al.*, 1945) resulting in very narrow β toxin zones on the plates. In this respect MUNCH-PETERSEN *et al.* (1945) and FEY (1953) considered about 3/4 of the sheep unsuitable, without giving information on the age of the animals. Plates prepared with washed cells of these unsuitable blood samples give excellent results.

2. **Basic medium of blood plates.** Ordinary meat broth, as often used for routine blood agar plates, did give rather small CAMP reactions. In search for a medium producing constantly large and clear reactions the following infusion broth was chosen.

I. 450 g of minced fresh ox heart are placed in 500 ml tap water and stored during 16 hours at 4 - 6°C., then during 1 hour at 50°C. with frequent stirrings; next it is brought at 100°C. for half an hour. The liquid is drained off with a sieve, then through filter paper. After cooling the fat is skimmed from the surface. Tap water is added till 500 ml. With 10% NaOH the pH is adjusted to 7.8. The liquid is brought at 100°C. during 5 min., then 10 g bactotryptose (Difco) are added.

II. To 50 ml tapwater 20 g bacto agar (Difco) and 5 g NaCl are added and dissolved at 110°C.

The hot preparations I and II are mixed, pH is adjusted to 7.4 followed by filtration through glasswool. Sterilisation during 20 min. at 110°C. Surplus can be stored at 4°C.

Sheep blood agar plates prepared with this heart infusion medium henceforth are called H-bloodplates.

3. The β toxin producing *Staphylococcus* strain is the same as used

by CHRISTIE in 1944. This strain, received from Dr J. I. TERPSTRA (Rijksseruminrichting, Rotterdam) labelled "Micrococcus", is referred to in this study as strain M.

4. 1276 strains of β haemolytic streptococci, isolated from human materials (mainly throat and rectum swabs) were grouped according to LANCEFIELD and examined for biochemical properties.

5. CAMP reactions of strains in our experiments were performed on H-bloodplates. Before use each batch of plates was controlled on suitability. A long straight narrow streak of strain M was inoculated over the middle of the plate. Of every *Streptococcus* culture a streak was applied just not touching the M streak and forming an angle of $35^\circ - 45^\circ$ with it (CHRISTIE, 1944 and MUNCH-PETERSEN, 1945). Each plate served for the reactions of 5 streptococcal strains. In each series of reactions performed on the same batch of plates, as a control a strain of known CAMP activity was included. After incubation at 37°C . for 18 till 24 hours the resulting configurations of β toxin zones and haemolysis in its various degrees were sketched with recordings of the important dimensions in mm. The CAMP reactions were noted as ++, +, \pm , and — on criteria which are explained later.

Some observations about the CAMP reaction in general.

By the above described method the areas of haemolysis in the positive CAMP reactions are shaped like candleflames (fig. 1). Just as in the before mentioned observations of MUNCH-PETERSEN the extension of the haemolysis in the β toxin zone is restricted. The curved borderline of the reaction comes very near the streak of growth of strain M, but never oversteps it. If both agents, β toxin and CAMP factor, would diffuse unhindered through the medium and the haemolytic reaction would take place in the area where the diffusion zones overlapped, a much larger reaction would result, indicated in fig. 1 by the dotted line. When certain coagulase negative staphylococci grow in a zone of β toxin, the produced shape of haemolysis is not like the CAMP reaction, but seems to be caused by regular diffusion of both components.

In order to explain the characteristic shape of the CAMP reaction various trials were taken repeatedly. Some outcomes may be presented here.

a) A streak of strain M is inoculated on a H-bloodplate and incubated

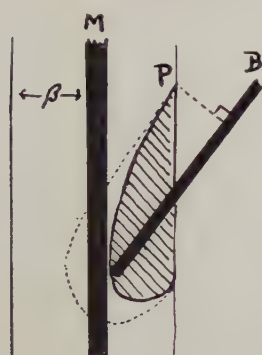


fig. 1

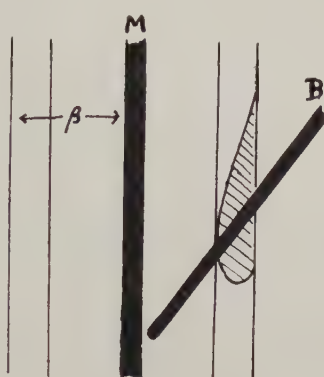


fig. 2

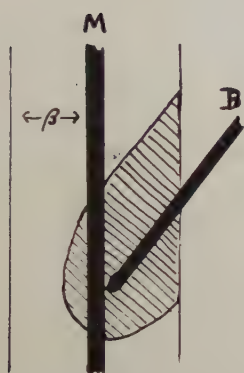


fig. 3

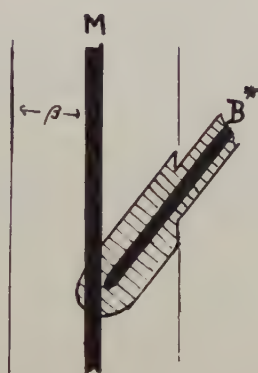


fig. 4

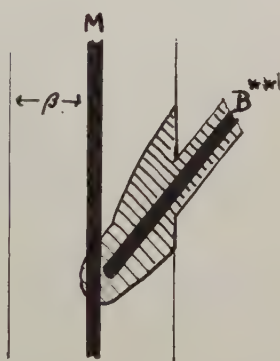


fig. 5

Fig. 1-5. M: growth of β -toxin producing strain M.

B: growth of group B streptococci. B*: growth of strongly haemolytic Camp negative streptococci. B***: growth of strongly haemolytic Camp positive streptococci. $\leftarrow \beta \rightarrow$ zone darkened by β -toxin. Shading: haemolytic areas.

P: see text.

at 37°C. for 18 hours. Then a streak of a group B *Streptococcus* strain is placed on the plate, which is incubated further till the next day. The result is given in fig. 2. The enlargement of the β toxin zone during the second period of incubation is divided from the zone of the first day by a sharp line, caused by the interruption of incubation. Only in the newly formed part of the β toxin zone a normal CAMP figure has developed.

b) If this test is reversed and the streak of the group B strain is inoculated first and the strain M 24 hours later, a large area with

complete haemolysis arises corresponding with the overlapping of two regular diffusion zones (fig. 3) and in extension agreeing with the dotted line in fig. 1.

c) According to trials in vitro β toxin combines strongly with sheep erythrocytes; by means of those the toxin can be removed easily out of a solution. On the contrary the CAMP factor in a solution has no affinity for blood corpuscles. We saw that erythrocytes in an agar medium, surrounded by CAMP factor, react to the adding of β toxin with maximal haemolysis. Haemolysis is hampered if erythrocytes in an agar medium have bound β toxin and CAMP factor is subsequently allowed to penetrate this medium. It seems that this hindrance becomes stronger, when the action of the β toxin on the erythrocytes has lasted longer. In some experiments where a small amount of dissolved β toxin had caused darkening of a part of the medium, and immediately afterwards group B streptococci had been inoculated on these areas, a small zone of complete haemolysis developed around the growth.

All trials indicate that the CAMP factor itself does not penetrate far in a β toxin zone, probably because it combines with the β toxin-erythrocyte complex without necessarily producing visible haemolysis. Contrarily the β toxin seems to be able to diffuse freely in a medium supplied with CAMP factor.

By mixing solutions of CAMP factor and of β toxin under certain conditions it is possible to obtain a fluid which causes a relatively broad zone of complete haemolysis when some drops of the mixture are brought in a hole on a H-bloodplate.

CAMP reactions of human strains of β haemolytic streptococci.

One may assume that the strength of the positive reactions is directly related to the distance of diffusion of demonstrable CAMP factor in the bloodplate. In the normal CAMP figure this distance is measured by the perpendicular from the top of the "candleflame" (point P in fig. 1) to the growth streak of the tested bacterial strain. The measure in mm of this line for the control strain in each series of reactions may serve as a unit for every test. On H-bloodplates this measure is usually 8 mm and it is not influenced by slight deviations of the angle between both streaks of inoculation and by the width of the β toxin zone. Each corresponding measured length acquired with a tested strain was expressed in a coefficient obtained by dividing this length with the unit. All coefficients

above 0.75 were noted as ++, from 0.75 and above 0.5 as +, from 0.5 and above 0.25 as \pm , from 0.25 and lower as negative. The results of this procedure on 1223 human strains of β haemolytic streptococci are tabulated in numbers of tested strains (table 1).

TABLE 1.
CAMP reactions in human β haemolytic streptococci.

CAMP reaction	Lancefield groups						percentages	
	A	B	C	F	G	ungrouped	A	B
++	60	205	—	—	1	—	29.7	94
+	57	11	—	—	—	—	28.2	5
\pm	44	1	1	—	—	—	21.8	0.5
—	41	1	231	296	195	79	20.3	0.5
number of strains	202	218	232	296	196	79	100.0	100.0

The group B strains have most constantly (99%) a positive (++) or (+) CAMP reaction. Negative and slightly positive reactions seem to be exceptions; both comprised only 0.5% in our material. The C, F, G and ungrouped (not belonging to A, B, C, F of G group) strains are predominantly negative, the positives being only 0.5% of the C and 0.5% of the G group.

A remarkably high percentage of positive reactions occurred amongst the A group streptococci; only 1/5 part was completely negative; an equal part showed slightly positive reactions. The rest (58%) however reacted frankly positive, half of them even strongly positive.

Nearly 1/4 of all group A strains exhibited a reaction as large and clear as the strongly positive reactions of the B strains (Table 2). Contrarily to the regular areas of complete haemolysis in the positive reactions of the B group strains, many of those of the group A strains had an irregular appearance, though it was always possible to distinguish clearly the upper part and the top of the "candleflame". In these cases parts of the area were incompletely haemolized and sometimes there were even defects in the "candleflame" figure.

On H-bloodplates these "incomplete" positive tests occurred in about half of the + and \pm reactions, and in about 1/5 of the ++ reactions. The kind of medium seems to be of influence for the demonstration of these incomplete, moderately positive reactions. In a limited number (53) of group A strains on routine bloodplates

TABLE 2.

Complete and incomplete CAMP reactions of group A streptococcal strains on H-bloodplates and on routine sheep blood agar plates (basic medium: ordinary meat broth).

CAMP-reaction		H-bloodplates		routine bloodplates	
		number strains	perc.	number strains	perc.
complete	+ +	47	23.3	9	20.9
	+	27	13.3	4	9.3
	±	23	11.4	1	2.3
incomplete	+ +	13	6.4	4	9.3
	+	30	14.9	—	—
	±	21	10.4	1	2.3
	—	41	20.3	34	55.9
		202	100.0	53	100.0

incomplete + or ± reactions were seen only once, whereas the strongly positive reactions occurred in a fairly high percentage; in total more than half of the strains reacted negative in this medium.

Especially the occurrence of incomplete tests amongst the strongly positive reactions of the A groups streptococci on H-bloodplates gives reason to suppose that the lytic agent of these streptococci, though related in properties, may more or less differ from the lytic agent (CAMP factor) of the group B streptococci.

It is very easy to read the CAMP reactions in streaks of group B streptococci. On the bloodplate outside the β zone these streaks have at most a narrow seam of incomplete haemolysis often mixed with some greening. Especially amongst the bovine group B strains α and β haemolysis may be even completely absent. In other groups, notably in the G group, many strains have along the streaks outside the β zones wider seams (max. 1.5 mm) of more intense till complete haemolysis. In these cases there is usually a considerable widening of the seam (max. till 3 mm) with complete haemolysis in the β toxin zone, resulting in an area of complete haemolysis running regularly along the streak of growth (fig. 4). Only with the group A strains frequently configurations arise which only can be explained as superpositions of CAMP figures on widened seams of haemolysis (fig. 5).

Some authors (MURPHY *et al.* 1952, FEY 1953) perform the CAMP reactions by streaking the streptococcal strain perpendicular to a streak of the β toxin producing staphylococci resulting in bowl-shaped positive tests. With this method it is much more difficult to distinguish slightly positive reactions from widened normal zones of haemolysis or from the haemolytic reactions of coagulase negative staphylococci. The principal advantage of inoculating both streaks under a sharp angle is the intensification of the typical shape of the CAMP reaction in the area of this angle, corrected by a smoothing of the reaction on the other side of the streak.

Application of the CAMP reaction in medical bacteriology.

In examinations of human materials for β haemolytic streptococci in general, anaerobical incubation of the bloodplates is of great advantage. Particularly colonies of group B streptococci grown under these conditions are easily recognizable. The rather large colonies are with very few exceptions moderately haemolytical, frequently pigmented (yellow till orange) and of a typical jelly-like consistency.

If a Gram-staining reveals Gram-positive cocci, a positive CAMP reaction furnishes the definite proof that the concerning bacteria are group B streptococci. Various materials are examined only aerobically in routine. Rather large unpigmented colonies, surrounded by a seam of very slight β haemolysis with some greening especially on the border of the seam, grown on aerobically incubated bloodplates may be group B streptococci. Finally these bacteria can be differentiated with certainty from other streptococci by means of the CAMP reaction. By this procedure we found *f.i.* in about 2½% of cases of cystitis or cystopyelitis, group B streptococci to be present in the urine samples.

In an extensive material, the results were controlled with LANCEFIELD'S grouping precipitation tests without finding exceptions to the above mentioned rules.

One should be aware of the occurrence of CAMP positive group A streptococci, which colonies are quite different from those of group B streptococci, and of some CAMP positive diphtheroid bacteria, either as contaminants or as members of the normal flora of throat or bowel.

CHEMICAL PART.

For the study of the chemical nature of the CAMP factor it was

necessary to obtain this factor in solution. Group B streptococci were grown in broth and subsequently filtered through Seitz E.K. filters.

First we used 5% horse blood broth, but blood products interfered with the chemical investigations; so it was necessary to use another medium. Todd Hewitt broth met the requirements, if glucose was substituted by maltose. After incubation for 18 hours (37°C.) the culture was centrifuged for 30 min. at 4000 p/m and was filtered through Seitz E.K. filters. In our experience the first 150 ml did not contain much CAMP factor because the filter adsorbed more or less factor. Crude fractions of CAMP factor were isolated from the filtrate in two different ways, resp. by precipitation with alcohol or ammonium sulfate. A further purification of the isolated fractions was obtained by dialysis against distilled water. Our results are given in table 3.

TABLE 3.

Fraction	Total N content mg %	CAMP activity
crude filtrate (I)	385	strong
dialysed crude filtrate (II)	156	strong
alcoholic precipitate of I (80% alc.)	63	moderate
alcoholic precipitate of II (60% alc.)	20	moderate
alcoholic precipitate of I (80% alc.) afterwards dialysed	11	absent
(NH ₄) ₂ SO ₄ precipitate of I (0.5 saturation) afterwards dialysed	24	moderate

The fraction obtained by alcoholic precipitation lost its CAMP activity after dialysis against distilled water. The fraction obtained by precipitation with ammonium sulfate was still active after dialysis. The purified fractions from the CAMP factor were more heat-labile than the crude filtrate. The CAMP activity of different fractions was in no way connected with the amount of polysaccharides

present. The polysaccharide estimation was performed according to WUNDERLY, 1954 (table 4).

TABLE 4.

Fraction	Intensity of polysaccharide staining	CAMP activity
crude filtrate (A)	positive	strong
alcoholic precipitate (B) of A (60% alc.)	positive	trace
alcoholic precipitate of the supernatant of B after addition of alcohol to 80%	negative	strong
(NH ₄) ₂ SO ₄ precipitate (0.5 saturation)	negative	strong

Therefore it is improbable that the CAMP factor has a polysaccharide structure. It follows that the CAMP factor is not related to group or type antigens, which are, as is well known, polysaccharides. However it must be stated that in purified extracts still a trace of polysaccharides is present.

The more purified active fractions were studied with paperchromatography using aqueous phenol as solvent. We saw three spots with average R.F. values resp. 0.10, 0.20 and 0.90. From comparison of paperchromatograms and CAMP activity of the different fractions we conclude that the CAMP factor belongs to the group of substances with R.F. value 0.90 ± 0.10 . Several determinations, total and amino nitrogen estimation (YEMM, 1955) before and after acid hydrolysis and paperchromatography of various hydrolysates, pointed in the direction of a polypeptidic structure of these substances.

Summary.

Detailed information concerning the performing and the reading of the CAMP reaction is given in this study. Comparable CAMP reactions were carried out on 1223 human streptococcal strains. Group B strains reacted positive in 99.5%; ++ 94%, + in 5% and

\pm in 0.5%. Group A strains were positive in about 80%; ++ in 30%, + in 28% and \pm in 22%. The reactions in the A group were often incomplete. There are reasons to suppose that the responsible agents in the two groups of streptococci are not quite identical. C and G group streptococci both reacted positive in only 0.5%; all tested F and ungrouped strains were negative. By adding the CAMP reaction to simple bacteriological methods it is possible to recognize with certainty human group B streptococci.

Some observations were made over the interaction of both components of the reaction, the β toxin and the lytic agent produced by B group streptococci (CAMP factor).

Preliminary chemical studies make it likely that the Camp factor has a polypeptidic structure.

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ACETOBACTER ESTUNENSE NOV. SPEC. AN ADDITION TO FRATEUR'S TEN BASIC SPECIES

by

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During a survey of *Acetobacter* occurring in apple juices, ciders and associated processing equipment, an organism was isolated which appeared to be *A. lovaniense* Frateur. Further examination, using the tests devised by FRATEUR (1950), revealed that in addition to the properties normally ascribed to *A. lovaniense*, this isolate, for which the name *A. estunense* is proposed, possessed the ability to produce cellulose. In view of this unusual property in an organism of the *Oxydans* group, a brief description of its other characteristics is given below.

METHODS.

Isolation and maintenance of this organism were carried out either on solid, or in liquid apple juice yeast extract medium (CARR, 1952). The media and reagents used for the classification of this organism were those described by FRATEUR (1950), while sugar utilisation and organic acid dissimilation tests had a basal medium consisting of casein hydrolysate, yeast extract and mineral salts at pH 4.8 and 4.2 respectively (CARR, 1957).

RESULTS.

Appearance of individual cells. A hanging drop culture of this organism showed mostly chains of ovoid cells which were non-motile. In a stained and fixed preparation these organisms were Gram-negative, circular to ovoid in shape, arranged singly, in pairs, in short chains of 5-6 cells and in irregular clumps.

Colonial form. Two types of colonies were observed on apple juice agar, but these were essentially the same except for size, one being 3 mm in diameter and the other less than 1 mm. Subsequent tests showed these two colony types to be biochemically homogeneous. By reflected light, individual colonies appeared to be circular with an entire margin; they had a convex elevation and a highly glossy surface. They appeared to consist of a matrix of clear substance in which were embedded islands of a buff coloured material. When touched on the surface with a wire loop, these colonies could be drawn into a short thread.

FRATEUR'S criteria. With the exception of cellulose production, the new organism showed the same reactions as those of *A. lovaniense*; these and other characteristics are compared in Table 1.

TABLE 1.
A comparison of *A. estunense* with *A. lovaniense*.

	<i>A. estunense</i>	<i>A. lovaniense</i>
Over-oxidation of ethanol	+	+
Catalase production	+	+
Growth in Hoyer's medium	+	+
Dihydroxyacetone from glycerol	—	—
Gluconic acid production	+	+
Cellulose production	+	—
Motility	—	+
Colonial form	Smooth and mucoid	Rough and irregular
Lactate metabolism	+	+

When *A. estunense* was first sub-cultured from a rich nutrient medium into Hoyer's medium, it took 7 days' incubation at 27°C. for a visible pellicle to develop. Subsequent transfers through Hoyer's medium, however, took a much shorter time to develop visible growth.

Cellulose production by this organism was much less consistent than from a culture of *A. xylinum* and it never produced a typical cartilaginous pellicle. Growth on solid media, such as apple juice agar and yeast extract glycerol agar, gave the typical cellulose reaction which could also be obtained with the pellicle from liquid apple juice medium. In contrast, the growth on liquid yeast extract glucose, beer, or Hoyer's medium, was non-cellulosic and friable.

Utilisation of sugars. The organism was tested against three sugars, namely glucose, fructose and sucrose, which were incubated for 7 days at 27°C. In addition, a culture was prepared which contained the basal medium without added carbohydrate. Sucrose and glucose supported growth, and acid was produced from both these sugars. Both the fructose medium and the basal medium alone supported growth, but in these the indicator (bromo cresol green) showed a rise in pH. Chromatographic analyses of the culture fluids, before and after growth had occurred, showed the following behaviour. Glucose disappeared, producing, in addition to acid, a trace of fructose and some unidentified carbohydrate material which was judged, by the position on the chromatogram, to be an oligosaccharide. Sucrose also disappeared and was replaced by acid, a considerable quantity of fructose and the unidentified substance formed from glucose. This suggests that the sucrose was inverted and only the glucose moiety metabolised. Fructose diminished slightly during the growth of the organism, otherwise no other change could be detected. In comparable tests using yeast extract – calcium carbonate agar as the basal medium, acid was produced only from glucose.

Organic acid dissimilation. This organism was tested against the following organic acids: malic, citric, lactic and succinic. The basal medium was as previously described and the cultures were shaken for 7 days at 27°C. They were likewise subjected to chromatographic analysis, which revealed that lactic acid had been completely metabolised, malic and succinic acid partially removed, while citric acid remained unchanged. No acidic end-products were detected as a result of the metabolism of these organic acids.

DISCUSSION

The discovery of this organism raises two important points. First, it has confirmed SHIMWELL's (1957) hypothesis that if a large number of *Acetobacter* species were examined, some would be discovered which differed from those described by FRATEUR (1950). Secondly, the question arises whether *A. estunense* should be regarded as a true species. This doubt has arisen because SHIMWELL (1956, 1957) has recently shown that most of the organisms of FRATEUR's *Mesoxydans*, *Oxydans* and *Peroxydans* groups are extremely mutable. SHIMWELL suggests that in view of these findings,

and taking into account priority of publication, the genus *Acetobacter* should contain two species, namely *A. aceti* (Pasteur) Beijerinck and *A. xylinum* (Brown) Beijerinck. The remaining organisms would then be regarded as mutants or variants of these two species.

Even if this suggestion were adopted, the fact remains that there are a number of different kinds of *Acetobacter* occurring in natural products, and, whether they are deemed to be species or not, FRATEUR's system provides a means of identifying them rapidly and easily. The name *A. estunense*, therefore, implies that this organism is distinct from any described by FRATEUR and it leaves the question open as to whether it is a true species.

A c k n o w l e d g e m e n t s.

I am much indebted to Dr J. L. SHIMWELL who confirmed my own observations on this organism and who also very kindly supplied additional information. I also wish to thank Dr G. C. WHITING, who carried out the chromatographic analyses mentioned in this paper, and Miss J. BIRD for technical assistance.

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ON THE PATHOGENESIS OF DIPHTHERIA

V. IMPORTANCE OF INTRAVENOUS INJECTION OF DIPHTHERIA SERUM¹⁾

by

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It may be regarded as an accepted fact that when a diphtheria infection is suspected the patient ought to be injected with antitoxic diphtheria serum without delay. It is indefensible to delay the serum injection until the result of the bacteriological examination is known. The bacteriological diagnosis should be only a confirmation of the bedside diagnosis made by the practitioner. A more detailed discussion of the desirability of the early injection of serum will be found in the publication by TASMAN and LANSBERG (1954).

Opinions differ, however, with regard to the best way of injecting this serum. It is generally deemed advisable to inject intramuscularly and only to resort to intravenous injection of the antitoxin, or some of it "in severe cases" (CHATTERJEE (1954), GORTER (1948), HYMAN (1948), CECIL and LOEB (1952), HERDESCHEE (1953) and CHRISTIE (1955)). LEPINTRE (1954) even goes so far as to maintain that the serum must never be administered intravenously but should always be injected intramuscularly or subcutaneously. LORENTZ (1953) suggests that the serum might be injected intraspinally, a method which, to the best of our knowledge, has never been adopted.

TASMAN, MINKENHOF, BRANDWIJK and SMITH (1954, 1955) have demonstrated that the saliva of diphtheria patients usually contains

¹⁾ Part I, II, III and IV: *Antonie van Leeuwenhoek* **19**, 135, 333, 1953; **20**, 417, 1954; **21**, 193, 1955.

TABLE 1.
Excretion of antitoxin into the saliva after intramuscular injection
of diphtheria serum.

No.	Antitoxin units injected	Time elapsed after injection of serum (hours)	Antitoxin units found per ml of saliva
1	40,000	4	none
		24	0.003
		120	0.017
2	30,000	10	none
		32	0.010
3	30,000	2	none
		15	none
		40	none
		120	0.004
4	40,000	1	none
		13	none
		37	none
		60	none
		96	0.050
5	20,000	0.5	none
		1	none
		5	none
		20	none
		30	none
		72	0.020
6	20,000	0.5	0.004
		1	0.004
		4	none
		6	none
		10	0.008
7	80,000	0.5	none
		1	none
		5	0.030
		7	0.030
		12	0.010
8	20,000	0.5	none
		1	none
		4	0.008
		6	0.004
		10	0.050
9	20,000	0.5	none
		1	none
		4	none
		6	none
		10	none
10	20,000	0.5	none
		1	none
		4	none
		6	none
		10	0.016
11	20,000	0.5	none
		1	none
		4	none
		6	none
		10	none

No.	Antitoxin units injected	Time elapsed after injection of serum (hours)	Antitoxin units found per ml of saliva
12	20.000	0.5	none
		1	none
		2	none
		6	none
		10	0.004
13	8.000	0.5	none
		1	none
		2	none
		6	none
		10	none
14	30.000	0.5	none
		1	none
		2	none
		4	none
		10	none
15	10.000	0.5	none
		1	none
		4	0.016
		6	0.084
		10	0.084
16	10.000	0.5	none
		1	none
		4	none
		6	0.016
		10	0.016

diphtheria toxin. The intramuscularly-injected purified diphtheria serum (obtained from horses) is sooner or later excreted in the saliva via the salivary glands and will then neutralize this toxin. Actively immunized persons do not excrete the (homologous) antitoxin in their blood through the salivary glands.

We thought it might be worth while to subject this excretion of antitoxin through the salivary glands to a closer examination, and more especially to try to ascertain whether there were any differences in this respect between patients who had been injected intramuscularly and those intravenously injected.

I. Antitoxin excretion in the saliva of patients treated with diphtheria serum.

The saliva of a number of patients was tested for antitoxin at various times after the intramuscular or intravenous injection of purified diphtheria serum. The results of this investigation are shown in tables 1 and 2. Table 3 summarizes the data given in these tables.

This excretion of antitoxin in the saliva via the salivary glands is

TABLE 2.
Excretion of antitoxin into the saliva after intravenous injection of
diphtheria serum.

No.	Antitoxin units injected	Time elapsed after injection of serum (hours)	Antitoxin units found per ml of saliva
1	10.000	0.5	0.006
		5	0.050
2	10.000	1	none
		6	0.030
3	10.000	0.5	0.030
4	10.000	0.5	0.080
5	10.000	0.5	0.008
		1	0.030
6	10.000	0.5	0.008
		1	0.008
7	10.000	0.5	0.016
		1	0.030
8	10.000	0.5	0.030
9	10.000	0.5	0.008
		1	0.016
10	10.000	0.5	0.004
		1	0.008
		4	0.040
11	20.000	0.5	0.017
		1	0.050
		4	0.050
		6	0.080
		10	0.080
12	20.000	0.5	0.017
		1.5	0.020
		3	0.020
		6	0.040
		11	0.050
13	10.000	0.5	0.030
		1	0.080
		2	0.080
		6	0.020
		10	0.020

very important. Our investigation showed that after only 30 minutes the saliva of all the intravenously injected patients except one already contained antitoxin and had therefore become a t o x i c¹⁾. In other words, from this moment no more toxin will

¹⁾ The first saliva samples were taken 30 minutes after the serum had been injected. It is by no means inconceivable that antitoxin may appear in the saliva of the intravenously injected patients even before that time.

TABLE 3.

Synopsis of the excretion of antitoxin into the saliva of intramuscularly and intravenously injected patients

A. Intramuscular injection

16 patients, injected with an average dose of 28,000 A.U.
per patient.

Of these 1 was positive after 30 min.

3 were " " 4—5 hours

3 " " " 10 hours

9 " " " 1—9 days

B. Intravenous injection

13 patients, injected with an average dose of 12,000 A.U.
per patient.

Of these 12 were positive after 30 min.

1 was " " 6 hours

be absorbed from the saliva which flows over the inflamed and damaged membranes of the tonsils and the pharynx. With the intramuscularly injected patients this rapid excretion of antitoxin was exceptional and as a rule it was a long time before the saliva contained any antitoxin. During this time diphtheria toxin can be absorbed from the saliva through the inflamed membranes of tonsils and pharynx.

This difference between the two groups of patients is the more striking as the intramuscularly injected patients received on an average 2.5 times as much antitoxin as those injected intravenously.

II. Blood serum curves.

We took samples of the blood of a number of patients before they received the diphtheria serum intravenously or intramuscularly and at set times after the injection, and determined the antitoxin titres. Table 4 gives the results found for 17 intramuscularly injected patients in blood samples taken before and 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22 days after the injection.

The absorption and excretion of intramuscularly injected antitoxin were found to vary greatly from one patient to another. As a rule, however, absorption after intramuscular injection was slow and the maximum serum titre was not reached until 2–6 days on an average had elapsed. A diagram of the average titre values, expressed in percentages of the maximum titre found, is given in figure 1.

TABLE 4.
Antitoxin content of the blood serum of intramuscularly injected patients

Patient No.	Diagnosis	Blood serum titre previous to serum injection A.U./ml	Dose of antitoxin in A.U.	Serum titre after the injection A.U./ml														Titre expressed in percentage of maximum titre found ¹⁾													
				Days elapsed after the injection														Days elapsed after the injection													
				1	2	4	6	8	10	12	14	16	18	20	22	1	2	4	6	8	10	12	14	16	18	20	22				
1	diphtheria	4	60,000	5.5	6.5	8.5	8.5	7.5	7.0	6.5	6.0	5.5	5.0	4.8	4.5			33	56	100	100	78	67	56	44	33	22	17	11		
2	diphtheria	<0.1	40,000	6.0	10.0	13.5	13.0	—	—	—	—	—	—	—	—			44	74	100	96	—	—	—	—	—	—	—	—		
3	tonsillitis	0.1	20,000	0.75	1.0	2.5	2.0	—	—	—	—	—	—	—	—			27	38	100	80	—	—	—	—	—	—	—	—		
4	tonsillitis	<0.1	40,000	1.75	3.0	4.0	—	3.0	2.0	1.0	—	0.75	0.5	—	0.2			44	75	100	—	75	50	25	—	19	13	—	5		
5	tonsillitis	<0.01	40,000	3.0	3.0	2.0	—	1.5	1.0	—	—	—	—	—	—			100	100	67	—	50	33	—	—	—	—	—	—		
6	tonsillitis	0.01	20,000	1.0	1.5	1.75	2.5	2.0	—	1.2	—	0.85	—	—	0.3			40	60	70	100	80	34	48	—	34	—	—	12		
7	tonsillitis	<0.01	20,000	0.3	0.6	1.2	1.3	1.0	0.7	—	0.4	0.3	—	0.27	0.24			23	46	92	100	77	53	—	30	23	—	20	18		
8	tonsillitis	<0.01	20,000	7.1	—	6.0	4.5	—	2.7	—	1.5	0.8	0.6	—	—			100	—	85	63	38	—	—	21	11	8	—	—		
9	diphtheria	0.02	20,000	2.3	3.0	3.0	2.3	1.5	—	—	1.3	1.1	—	—	—			77	100	100	77	50	—	43	37	—	—	—	—		
10	tonsillitis	<0.01	20,000	1.25	3.0	3.6	2.5	2.0	1.8	—	1.4	—	0.7	—	—			35	83	100	70	56	50	—	40	—	20	—	—		
11	diphtheria	<0.01	80,000	7.6	16.0	25.0	16.0	13.0	10.0	6.0	—	3.5	2.4	—	—			30	64	100	64	50	40	24	—	14	10	—	—		
12	tonsillitis	0.02	20,000	1.0	2.0	2.5	2.5	1.73	—	0.9	—	0.7	—	0.65	—			50	80	100	100	70	—	36	—	28	—	26	—		
13	tonsillitis	0.08	80,000	2.3	10.0	8.5	—	4.0	3.4	3.4	—	1.8	—	—	0.9			23	100	85	—	40	34	34	—	18	—	—	9		
14	asthma	<0.01	20,000	3.0	4.8	5.0	3.2	2.7	2.0	1.2	—	0.68	—	0.45	—			60	96	100	64	54	40	24	—	14	—	9	—		
15	cutaneous diphtheria	<0.01	20,000	1.0	1.7	2.2	1.5	1.2	—	0.81	—	0.67	—	—	0.24			45	77	100	70	54	—	37	—	30	—	—	11		
16	tonsillitis	0.04	20,000	1.5	2.7	1.25	1.25	1.25	1.25	—	0.68	—	0.6	—	0.6			56	100	46	46	46	—	25	—	25	—	25	—		
17	diphtheria	<0.01	8,000	2.3	3.4	2.9	2.1	1.6	1.1	0.75	0.7	0.5	0.3	—	—			67	100	85	60	47	32	22	22	15	9	—	—		
																		Average:	50	78	90	78	59	44	33	(33)	23	(13)	(19)	(11)	

¹⁾ In these calculations the titre values found before the injection of serum have been subtracted from those after the injection, our object being to ascertain the changes subsequent on the injection of heterologous diphtheria antitoxin.

TABLE 5.
Antitoxin content of the blood serum of intravenously injected patients.

Patient No.	Diagnosis	Blood serum titre previous to serum injection A. U./ml	Dose of anti-toxin in A. U.	Serum titre after the injection, A. U./ml														Titre expressed in percentage of maximum titre found ¹⁾																		
				Days elapsed after the injection														Days elapsed after the injection																		
				1	2	4	6	8	10	12	14	1	2	4	6	8	10	12	14	1	2	4	6	8	10	12	14									
1	tonsillitis	0.10	10,000	4.5	4.0	2.5	2.0	2.0	2.0	2.0	1.75	0.75	2.0	2.0	0.5	0.35	0.25	0.1	0.75	100	89	55	43	43	43	37	17	100	100	100	100	63	33	13	—	—
2	tonsillitis	<0.10	10,000	—	1.75	1.25	0.75	0.5	0.35	0.25	0.1	0.5	0.35	0.25	0.1	0.75	0.25	0.1	0.75	—	100	71	43	28	20	14	6	—	—	—	—	—	—	—	—	—
3	tonsillitis	<0.10	10,000	1.5	—	1.0	0.55	0.45	0.45	0.45	0.3	0.45	0.45	0.45	0.3	0.45	0.3	0.3	100	—	66	37	30	30	30	20	100	100	100	100	66	37	30	30	20	
4	tonsillitis	0.75	10,000	3.0	2.5	2.0	2.0	1.75	1.75	—	1.25	1.75	1.75	1.75	1.25	1.75	1.25	1.25	100	78	56	56	44	44	—	22	100	100	100	100	56	44	44	—	—	
5	tonsillitis	0.75	10,000	3.0	2.5	2.0	2.0	1.75	1.75	—	1.0	0.85	1.0	0.85	1.0	0.85	0.85	0.85	100	78	56	56	44	44	—	4	100	100	100	100	56	44	44	—	—	
6	tonsillitis	0.25	10,000	3.0	2.5	2.0	2.0	2.0	2.0	—	0.7	0.7	—	—	—	—	—	—	100	82	64	64	—	—	25	—	100	100	100	100	64	64	—	—	—	
7	tonsillitis	<0.10	10,000	2.0	2.0	—	—	1.25	0.65	0.25	—	0.65	0.65	0.25	—	—	—	—	100	100	—	—	—	—	33	13	—	100	100	100	100	—	—	—	—	—
8	tonsillitis	0.20	10,000	3.0	1.75	1.75	1.25	1.25	—	—	—	0.5	—	—	—	—	—	—	100	55	55	37	22	17	13	—	100	100	100	100	37	22	17	13	—	
9	diphtheria	<0.01	10,000	4.5	2.5	2.0	1.5	1.0	0.75	0.6	—	0.75	0.6	0.5	0.4	—	—	—	100	64	44	44	30	24	20	16	—	100	100	100	100	44	30	24	20	16
10	diphtheria	<0.01	10,000	2.5	1.5	1.0	0.75	0.6	0.5	0.4	—	0.75	0.5	0.4	—	—	—	—	100	60	40	40	30	24	20	16	—	100	100	100	100	40	30	24	20	16
11	tonsillitis	<0.01	10,000	4.0	2.5	1.5	1.0	0.75	0.5	0.25	0.25	0.75	0.5	0.25	0.25	0.25	0.25	0.25	100	62	38	38	25	19	13	7	100	100	100	100	38	25	19	13	7	
12	diphtheria	<0.01	10,000	1.8	—	1.0	0.8	0.5	0.3	0.2	0.1	0.8	0.5	0.3	0.2	0.1	0.1	0.1	100	—	56	44	28	17	11	5	100	100	100	100	56	44	28	17	11	
13	tonsillitis	<0.01	10,000	3.45	3.4	3.4	2.0	1.0	1.0	0.7	0.55	1.0	1.0	0.7	0.55	1.0	0.7	0.55	100	98	98	98	58	29	21	16	100	100	100	100	98	58	29	21	16	
14	tonsillitis	0.02	10,000	3.4	3.0	—	—	1.25	0.8	—	0.6	1.25	0.8	—	—	—	—	0.6	100	90	—	37	37	24	—	17	100	100	100	100	37	24	—	—	—	
15	tonsillitis	1.0	10,000	3.0	3.0	1.5	1.5	1.5	—	—	1.2	1.5	—	—	—	—	1.2	1.2	100	100	25	25	25	—	10	100	100	100	100	25	25	—	—	—		
16	tonsillitis	<0.01	10,000	1.5	—	1.0	—	—	0.45	0.3	0.26	1.0	—	—	—	—	0.3	0.26	100	—	67	—	—	30	20	18	100	100	100	100	67	—	—	—	—	
17	diphtheria	<0.01	10,000	3.71	3.4	1.5	1.5	1.0	0.68	0.5	0.32	1.0	0.68	0.5	0.32	1.0	0.68	0.5	100	91	40	40	40	27	18	9	100	100	100	100	40	40	27	18	9	
18	tonsillitis	0.40	10,000	4.0	2.3	1.5	1.0	0.75	0.6	0.56	0.56	0.75	0.6	0.56	0.5	0.56	0.56	0.5	100	50	30	30	17	10	6	4	3	100	100	100	100	30	17	10	6	4
19	tonsillitis	<0.01	20,000	3.71	2.2	1.5	1.25	1.0	0.7	0.45	0.35	1.0	0.7	0.45	0.35	1.0	0.7	0.45	100	60	40	40	34	27	18	12	10	100	100	100	100	40	34	27	18	12
20	diphtheria	<0.01	10,000	2.7	1.5	1.25	1.0	0.68	0.5	0.3	0.35	1.0	0.68	0.5	0.3	0.35	1.0	0.68	100	56	46	46	37	25	19	13	100	100	100	100	46	37	25	19	13	
											Average:								100	77	53	39	31	23	16	12										

¹⁾ In these calculations the titre values found before the injection of serum have been subtracted from those after the injection, our object being to ascertain the changes subsequent on the injection of heterologous diphtheria antitoxin.

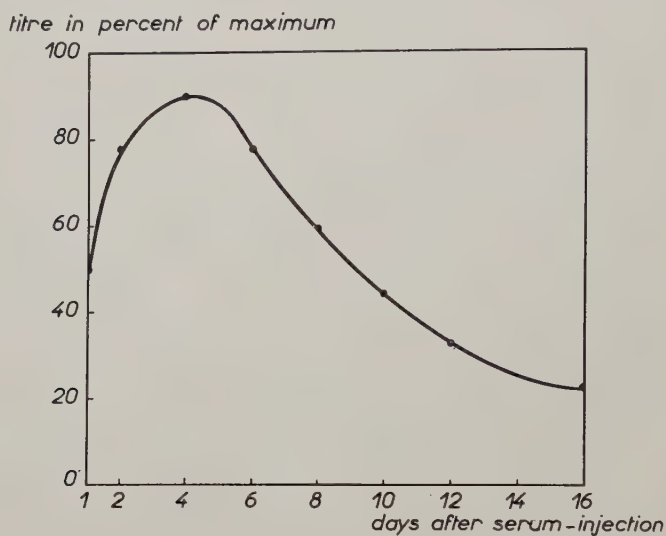


Fig. 1. Average serum titres after intramuscular injection of serum, expressed in percentage of the maximum titre found.

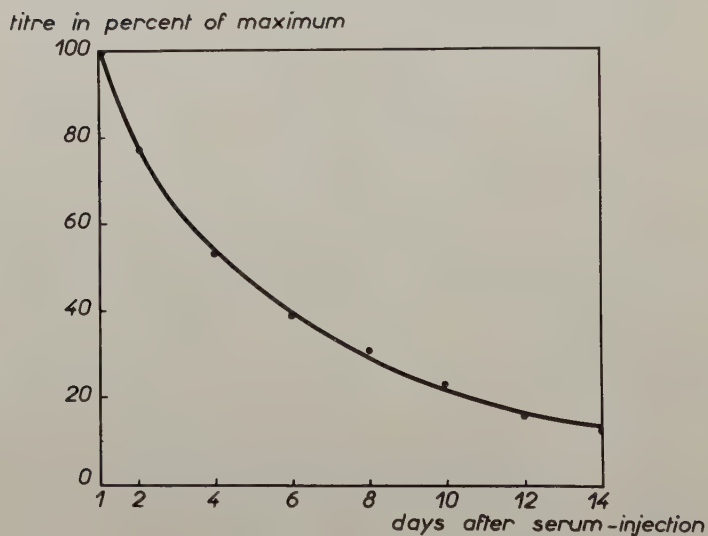


Fig. 2. Average serum titres after intravenous injection, expressed in percentage of the maximum titre found.

Table 5 shows the results for 20 intravenously injected patients whose antitoxin titres were determined before and 1, 2, 4, 6, 8, 10, 12 and 14 days after the injection.

The intravenous injection of antitoxin of course brings the patients serum "up to titre" immediately. As was the case with the intramuscularly injected patients, the titres found for the individual patients after intravenous injection varied considerably. Figure 2 represents the average serum titres, the maximum serum titre found (one day after the injection) being taken as 100%.

One is immediately struck by the great similarity between the two serum curves. This is brought out even more clearly, when the percentage serum titres (expressed as percentages of the maximum titre found) are plotted on a logarithmic scale and in both cases the average titre found four days after the injection is taken as 100% ¹⁾.

Table 6 and figure 3 give the results of these calculations. In the latter the two lines practically coincide from the fourth day after the serum injection.

TABLE 6.

Logarithms of the mean serum titres after the intravenous or intramuscular injection of serum, assuming the serum titre found four days after the injection to be 100%

Days elapsed after the injection of serum	Logarithms of the mean serum titres	
	Intravenous injection	Intramuscular injection
1	2.28	1.74
2	2.16	1.94
4	2.00	2.00
6	1.87	1.94
8	1.78	1.82
10	1.63	1.69
12	1.48	1.56
14	1.36	—
16	—	1.41

From this we may conclude that the intravenously injected serum is not excreted more rapidly than the intramuscularly administered antitoxin, so that in this respect there is no reason

¹⁾ On an average the absorption of intramuscularly injected serum is finished at the end of this period. The excretion of this intramuscularly injected antitoxin which then takes place is strictly comparable with that of the intravenously injected serum.

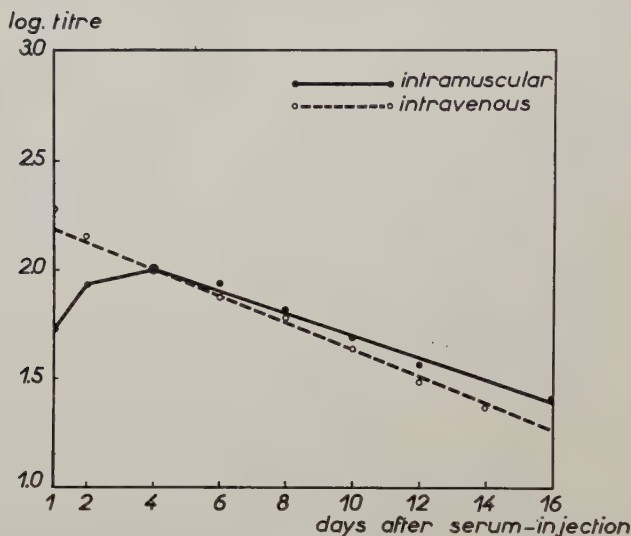


Fig. 3. Logarithms of the average serum titres after intravenous and intramuscular injection, expressed in percentages of the titres found four days after the injection.

why the intramuscular injection of serum should be preferred to the intravenous method. As long ago as 1936 the Danish investigator MADSEN arrived at the same conclusion after a similar investigation.

In view of the above results, the obvious thing would have been to test the presumptive superiority of the intravenous method as compared with the intramuscular injection on a large number of patients. However, such a plan encounters many difficulties, the chief obstacle being the small number of diphtheria patients available under the present circumstances.

For this reason we confined ourselves to a number of model experiments with laboratory animals; in other words we evolved and applied an "animal model" for these problems.

III. Design and application of the "animal model".

When a guinea-pig is injected with a certain number of lethal doses of diphtheria toxin, it can be saved from death or protected from typical morbid symptoms by injecting antitoxin previous to or shortly after the toxin injection. When the quantities of the toxin and antitoxin to be injected have been fixed, the interval between

the toxin injection and the administration of antitoxin will determine the clinical picture. If a short interval is chosen, the animals thus treated will show only very slight and transient symptoms or none at all. If a long interval is allowed, most animals will show severe manifestations (marked loss of weight, severe cardiac manifestations and paralyses) or the majority of them will die as a result of this treatment.

In other words, therefore, a suitable choice of the three determining factors, *viz.*, the amount of toxin, the amount of antitoxin and the length of the interval between the two injections, will make it possible to control the clinical picture in such a way that special attention can be given to different aspects of it.

A well-standardized "animal model" of this kind will also provide an excellent means of ascertaining experimentally the influence of a fourth factor, *i.e.* the route by which the antitoxin is injected.

After some preliminary experiments, we used male¹⁾ guinea-pigs, weighing from 300 to 350 g, in all our further investigations. These were injected subcutaneously with 5 MLD of diphtheria toxin at the beginning of each experiment. A certain number of hours afterwards the animals were injected with 1 A.U. of diphtheria antitoxin, half of them receiving this antitoxin intramuscularly and the other half intravenously. By determining beforehand the time required for the different injections, it was possible to ensure that the time between the toxin and the antitoxin injection was the same for every animal in each separate experiment. In each experiment a number of guinea-pigs was injected with toxin only without a subsequent antitoxin injection, as a control of the toxin dose. These control animals were always dead on the second or third day after toxin injection.

First animal experiment.

Two groups of guinea-pigs received 5 MLD of diphtheria toxin subcutaneously; after 9 hours 1 A.U. was injected intramuscularly into 15 animals and intravenously in 15 others. Of the intramuscularly injected guinea-pigs 4 died, after 2, 6, 7 and 9 days re-

¹⁾ The intravenous injection of serum in the male guinea-pig is technically much simpler than in the female animal. The serum was injected into a branch of the femoral vein in the subcutis of the inside of the thigh.

spectively, while 4 of the intravenously injected animals died after 6, 7, 8 and 10 days respectively. All the animals were weighed twice every day, after which the mean weight of each group was calculated. Table 7 gives the results of this experiment, which are graphically represented by the diagram in figure 4. The initial weight per group is here taken as 100.

TABLE 7.

Changes in the weight of guinea-pigs intravenously or intramuscularly injected with diphtheria antitoxin.

Days elapsed after the injection of serum	Mean weight of guinea-pigs on consecutive days	
	intravenously injected	intramuscularly injected
0	306 g = 100 %	307 g = 100 %
1	316 g = 103 %	307 g = 100 %
2	308 g = 101 %	299 g = 97.4%
3	305 g = 99.7%	291 g = 94.8%
4	291 g = 95 %	286 g = 93.1%
5	294 g = 96 %	281 g = 91.5%
6	293 g = 95.7%	275 g = 89.6%
7	289 g = 94.4%	271 g = 88 %
8	289 g = 94.4%	262 g = 85.3%
9	286 g = 93 %	255 g = 83 %
10	289 g = 94.4%	255 g = 83 %
11	293 g = 95.7%	256 g = 83.3%

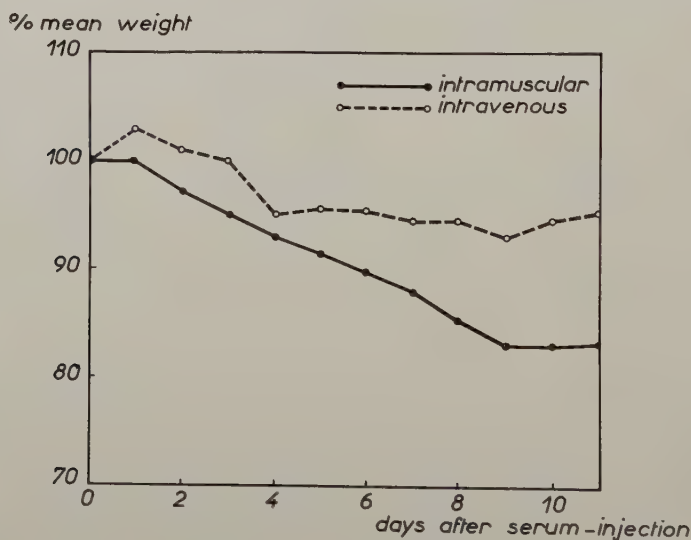


Fig. 4. Weight of guinea-pigs after intramuscular or intravenous injection of diphtheria antitoxin.

From these results we may draw the following conclusions. The guinea-pigs that had been injected intramuscularly with serum showed a greater and more prolonged loss of weight than the intravenously injected animals. Therefore the general condition of the former throughout the experiment was considerably worse than that of the latter under otherwise identical conditions.

Second animal experiment.

If the interval between toxin injection and antitoxin injection is prolonged, one may expect a greater number of deaths in both groups of animals. In order to test the difference between the intramuscular and the intravenous injection of serum, two groups of 65 guinea-pigs were now subcutaneously injected with 5 MLD of diphtheria toxin. After $9\frac{1}{2}$ hours they were given 1 A.U. of antitoxin.

TABLE 8.

Mortality among guinea-pigs intravenously or intramuscularly injected with diphtheria antitoxin, diphtheria toxin having been injected $9\frac{1}{2}$ hours before. Number of animals in each group 65; toxin dose 5 MLD; antitoxin dose 1 A.U.

Days elapsed after injection of serum	Deaths in each group, per day	
	serum injected intravenously	serum injected intramuscularly
1	0	7
2	0	0
3	1	1
4	7	6
5	7	6
6	5	4
7	3	5
8	2	5
9	1	2
10	1	6
11	2	4
12	0	6
13	4	2
14	0	1
15	1	1
Total	34	56
	Out of 65 animals 34 died and 31 survived	Out of 65 animals 56 died and 9 survived

One group was injected intravenously with the antitoxin, the other group receiving the antitoxin intramuscularly. The results of this experiment are given in table 8.

These figures suggest that guinea-pigs previously treated with toxin are given a considerably better chance of survival by the intravenous serum injection than by the intramuscular injection of the same dose of diphtheria antitoxin.

Third animal experiment.

It was to be expected that the use of a considerably shorter interval between the injection of toxin and of antitoxin would result in a reduced number of deaths and would also provide information as to the effect of the method of serum administered on the paralytic phenomena which appear in a later stage of the disease.

TABLE 9.

Incidence of paralysis among guinea-pigs intravenously or intramuscularly injected with diphtheria antitoxin, diphtheria toxin having been injected $6\frac{1}{2}$ hours previously. Number of animals in each group: 46 and 48 respectively; toxin dose 5 MLD; antitoxin dose 1 A.U.

Days elapsed after in- jection of serum	intravenous		intramuscular	
	Number of animals suffer- ing from paralysis	Percentage of total number of animals	Number of animals suffer- ing from paralysis	Percentage of total number of animals
15	0	0	0	0
16	0	0	1	2
17	0	0	2	4
18	0	0	6	12.5
19	0	0	11	23.0
20	0	0	19	39.6
21	3	6.5	24	50.0
22	4	8.8	26	56.2
23	8	17.4	29	60.4
24	10	21.7	31	64.6
25	11	24.0	35	73.0
26	11	24.0	39	81.3
27	11	24.0	39	81.3
28	12	26.1	43	89.6
29	12	26.1	44	91.7
30	14	30.5	47	98.0
31	14	30.5	47	98.0
32	15	32.6	48	100.0

For this purpose, two groups of 46 and 48 guinea-pigs respectively were given 5 MLD of toxin subcutaneously after which they were intravenously or intramuscularly injected with 1 A.U. of antitoxin $6\frac{1}{2}$ hours later. Table 9 shows the incidence of paralysis. This primarily and chiefly affected the hind legs. Figure 5 is a diagram of these results.

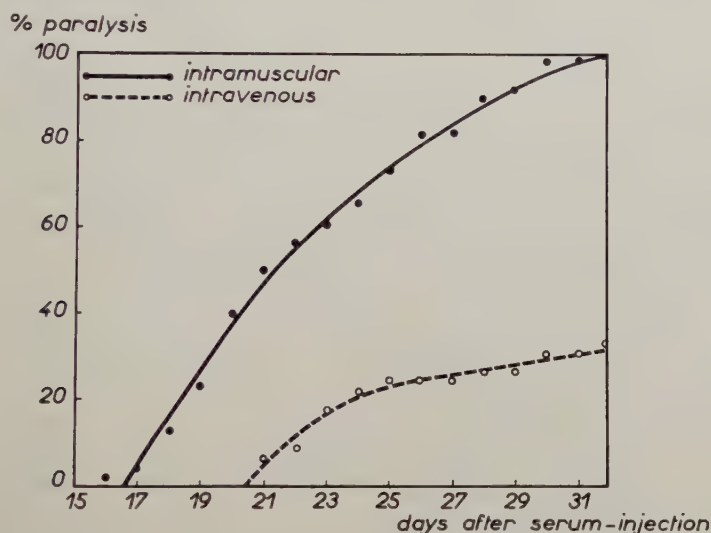


Fig. 5. Incidence of paralysis among guinea-pigs intramuscularly or intravenously injected with diphtheria antitoxin.

These data show that – under the given conditions of the experiment – paralysis occurred fairly late: after 16 days among the intramuscularly injected animals, and not until after 21 days among the animals that had been given an intravenous serum injection. Thirty-two days after the beginning of the experiment 100 per cent of the intramuscularly treated guinea-pigs showed unmistakable signs of paralysis, whereas this percentage was only 33 among the intravenously injected animals.

It thus appears that the intravenous injection of serum very considerably reduces the chance of occurrence of post-diphtheritic paralyses. These paralyses also appear at a later time than is the case after intramuscular injection of serum.

Fourth animal experiment.

It is a well-known fact that the heart is the organ that suffers most from the harmful effect of diphtheria toxin. Therefore it goes

without saying that in our efforts to design a well-planned experiment we paid full attention to these affections of the heart.

For this purpose two groups of 184 guinea-pigs were subcutaneously injected with 5 MLD of diphtheria toxin. After $6\frac{1}{2}$ hours they all received 1 A.U. of antitoxin, the first group intravenously and the second intramuscularly. After 1, 3, 6, 9, 12 and 15 days respectively, about $\frac{1}{6}$ of the total number of animals per group were killed, and the hearts removed and fixed in 20% aqueous formalin solution. The animals that had died spontaneously at the times mentioned were regarded as "killed" and their hearts treated in the same way. Frozen sections were made of roughly the same part of the wall of the left ventricle of each heart. For the study of the myocardial lesions these sections were stained with haematoxylin-eosin. For the demonstration of fatty degeneration separate sections were stained with Sudan III.

To provide a standard of comparison for the microscopically observed histological changes in the hearts of the injected animals, we sacrificed 4 normal, non-treated guinea-pigs per group of killed animals and spontaneous deaths. The hearts of these animals were prepared in the same way.

This pathological study was organized in such a way that the member of our team who performed the dissections and the microscopic examination (VINK) never knew in advance to which category (injected or non-injected) the various guinea-pigs belonged. Thus an objective appreciation of the material was ensured.

The changes observed ranged from a very slight local infiltration with mononuclear cells to a strong, practically diffuse, infiltration with many mononuclear cells, sometimes accompanied by leucocytes, lymphocytes, or fibroblasts. In slight cases the fatty degeneration of the myocardium consisted in a small number of fatty particles in only a few muscle-fibres. In strongly degenerated hearts practically all the fibres contained numerous, sometimes fairly large, drops of fat.

In this experiment also a number of control animals were injected with toxin only and not with serum. All these animals died within two days, their hearts always showing considerable fatty degeneration.

The gravity of the lesions microscopically observed in the hearts of the different animals varied greatly and was (more or less quantitatively) classified as \pm , +, ++ or +++.

TABLE 10.

Distribution of lesions of the heart (myocarditis and fatty degeneration combined) found among the various groups of guinea-pigs. All animals were given 5 MLD of toxin subcutaneously, followed by an injection of 1 A.U. of antitoxin, intramuscular or intravenous, after 6½ hours.

Number of days between serum injection and killing	Intravenously injected			Intramuscularly injected		
	Number of animals killed	total number of heart lesions	% of heart lesions	Number of animals killed	total number of heart lesions	% of heart lesions
1	30	1	3.3	30	9	30.0
3	35	19	54.3	38	16	42.1
6	32	16	50.0	31	22	71.0
9	29	18	62.1	30	25	83.3
12	29	7	24.1	30	17	56.7
15	29	9	31.0	25	10	40.0

TABLE 11.

Distribution of serious lesions of the heart found among the various groups of guinea-pigs. All animals were given 5 MLD of toxin subcutaneously, followed by an injection of 1 A.U. of antitoxin, intramuscular or intravenous after 6½ hours.

Number of days between serum injection and killing	Intravenously injected			Intramuscularly injected		
	Number of animals killed	total number of heart lesions	% of heart lesions	Number of animals killed	total number of heart lesions	% of heart lesions
1	30	0	0	30	1	3.3
3	35	5	14.3	38	6	15.9
6	32	5	15.6	31	9	29.0
9	29	9	31.0	30	17	56.6
12	29	3	10.3	30	10	33.3
15	29	2	7.0	25	3	12.0

Occasionally the non-injected control animals (neither toxin nor serum) also showed slight lesions of the myocardium, classifiable as \pm or $+$. This was the case in 15 of the 24 normal control animals. Cases of fatty degeneration of the heart were never found among the non-treated animals.

The complete results of this experiment are shown in table 10, which therefore includes all the lesions of the heart (mild and severe cases combined).

As stated above we occasionally found slight cardiac lesions classified as \pm or $+$, in non-treated guinea-pigs. Some of the treated animals also showed such slight lesions. It seemed only logical to leave these slight lesions, also found in non-treated animals, out of account. Consequently table 11 only includes the grave lesions (myocarditis and fatty degeneration taken together). The data of table 11 are represented graphically in figure 6.

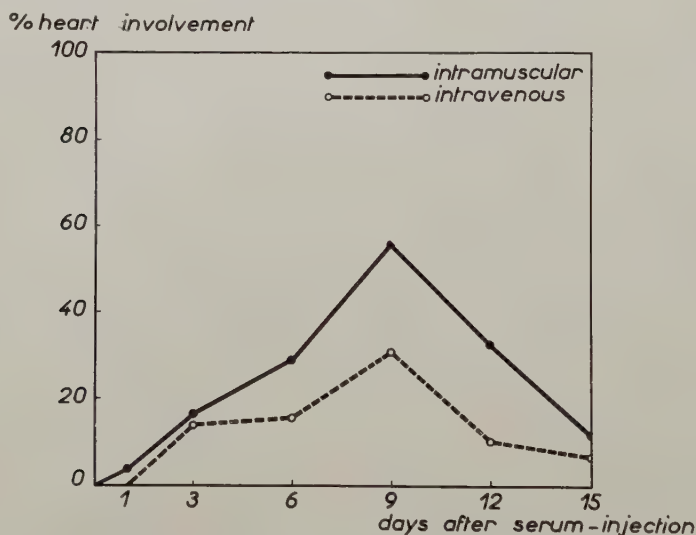


Fig. 6. Cardiac lesions (myocarditis and fatty degeneration combined) observed in guinea-pigs after intramuscular or intravenous injection of diphtheria serum.

More or less severe lesions of the heart (myocarditis and fatty degeneration) occurred in both groups. This is understandable as all the animals had been exposed to the injurious action of 5 MLD of diphtheria-toxin for $6\frac{1}{2}$ hours, before they were injected with the antitoxin.

Under the given experimental conditions, these lesions reached their climax after nine days, recovery then setting in at the same time in both groups. Of course this last circumstance may have been due to chance. It is not impossible that if the experiment were arranged differently the times of recovery would not coincide.

Finally we tried to divide the observed lesions into myocarditis and fatty degeneration.

Table 12 shows the incidence of myocarditis in the two groups (intravenously or intramuscularly injected with antitoxin), the figures between brackets indicating how many animals also suffered from fatty degeneration.

The figures given in table 12 for lesions of the myocardium do not include very slight lesions such as were also found in some of the control animals. Fig. 7 is a graphical representation of the data of table 12.

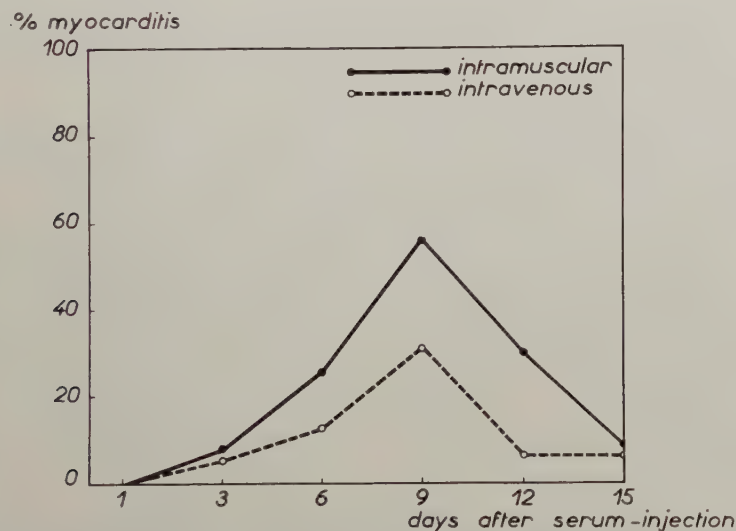


Fig. 7. Severe lesions of the myocardium found in guinea-pigs after intramuscular or intravenous injection of diphtheria serum.

Table 13 shows the distribution of the cases of fatty degeneration, the figures between brackets this time indicating the number of animals that also showed definite symptoms of myocarditis. Figure 8 is a graph of the data of table 13.

With respect to the observed lesions of the heart, there again emerges a clear difference between the results of the two methods,

TABLE 12.

Distribution of cases of myocarditis found among the various groups of guinea-pigs. All animals were given 5 MLD of toxin subcutaneously, followed by an injection of 1 A.U. of antitoxin, intramuscular or intravenous, after 6½ hours.

Number of days between serum injection and killing	Intravenously injected			Intramuscularly injected		
	Number of animals killed	total number of myocarditis	% of myocarditis	Number of animals killed	total number of myocarditis	% of myocarditis
1	30	0 (0)	0	30	0 (0)	0
3	35	2 (0)	5.7	38	3 (2)	8.0
6	32	4 (1)	12.5	31	8 (6)	25.8
9	29	9 (0)	31.0	30	17 (6)	56.7
12	29	2 (0)	6.9	30	9 (3)	30.0
15	29	2 (0)	6.9	25	2 (1)	8.0

The figures between brackets refer to the number of animals also suffering from fatty degeneration.

TABLE 13.

Distribution of cases of fatty degeneration found among the various groups of guinea-pigs. All animals were given 5 MLD of toxin subcutaneously, followed by an injection of 1 A.U. of antitoxin, intramuscular or intravenous, after 6½ hours.

Number of days between serum injection and killing	Intravenously injected			Intramuscularly injected		
	Number of animals killed	total number of fatty degeneration	% of fatty degeneration	Number of animals killed	total number of fatty degeneration	% of fatty degeneration
1	30	0	0	30	4 (2)	13.3
3	35	12 (4)	34.3	38	12 (5)	31.6
6	32	3 (1)	9.4	31	12 (11)	38.7
9	29	0	0	30	7 (7)	23.3
12	29	2 (1)	6.9	30	4 (2)	13.3
15	29	2 (0)	6.9	25	3 (2)	12.0

The figures between brackets refer to the number of animals also suffering from myocarditis.

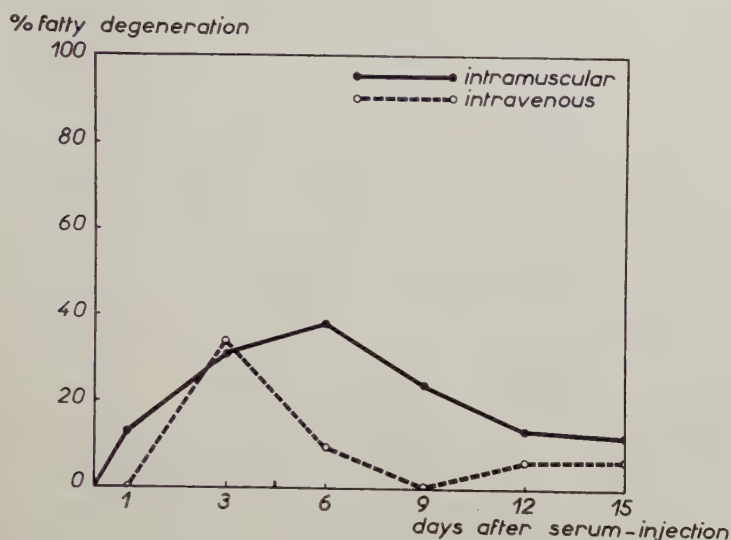


Fig. 8. Cases of fatty degeneration of the heart found among guinea-pigs after intramuscular or intravenous injection of diphtheria serum.

which is strongly in favour of the intravenous injection of serum. This is easily accounted for. After the intravenous antitoxin injection the serum of the animal is practically immediately "up to titre", i.e. the total amount of antitoxin is available straightaway for neutralization of the toxin still circulating freely at that moment. This can then no longer cause a lesion of the heart. Maybe part of the toxin which has already penetrated into the myocardium is also neutralized.

The effect of the intramuscular injection of serum is the same, but slower, because of the comparatively slow absorption of the antitoxin thus injected. The toxin still circulating freely is rendered inactive more slowly or can act longer upon the myocardium with all the consequences of this.

Summary of results of the animal experiments.

Groups of guinea-pigs were subcutaneously injected, two at a time, with a constant amount of diphtheria toxin (5 MLD). After a certain number of hours these animals all received a constant amount of antitoxin (1 A.U.) half of them being injected intravenously and

the other half intramuscularly. The intravenous injection of serum was found to have a considerably greater therapeutic effect than the intramuscular method. The animals injected intravenously with serum showed:

- 1) less loss of weight and a more rapid recovery of weight,
- 2) fewer deaths,
- 3) less frequent and later occurrence of paralysis and
- 4) fewer lesions of the heart (myocarditis and fatty degeneration) than the intramuscularly injected animals.

IV. General observations.

Before discussing the significance of these animal experiments with respect to the treatment of the human diphtheria patient, we may here add some remarks on this "animal model".

The experiments with guinea-pigs described in detail above were performed under strictly standardized conditions. The doses of toxin and antitoxin injected were the same for all animals. The animals were all of the same sex and had the same weight per group. The time between the injection of toxin and of antitoxin was the same for each pair. In this way we found a quantitative difference between the therapeutic effects of the intravenous and the intramuscular injection of serum. Had the determining factors for these experiments, *viz.*, amount of toxin, amount of antitoxin and interval between the two injections, been chosen differently, we should no doubt have seen the same qualitative differences, but the absolute figures would not have been the same.

Obviously, the quantitative results obtained with this animal model cannot be quantitatively transferred to the therapy of human diphtheria patients. The criteria for the latter group are different from those applied in the animal experiment.

Assuming, however, that the pathogenesis of human diphtheria is determined by the formation of diphtheria toxin by the *C. diphtheriae* infecting the patient and by the effects of this toxin, especially on the heart and the nervous system, we may indeed apply the qualitative results obtained with this laboratory experiment to man.

Summarizing the results given under I, II and III, we may therefore conclude that for the treatment of human diphtheria also the intravenous injection of serum is much to be preferred to the intramuscular route for the following reasons:

1. After the intravenous injection of antitoxin the patient's serum is almost immediately "up to titre"; in other words the total amount of diphtheria antitoxin is transported very quickly through the blood to the organs concerned. Intramuscularly injected serum is absorbed only slowly. On an average this absorption is not completed until after four days, but it may take as much as six days.
2. As a non-specific protein, the intravenously injected antitoxin is not broken down and excreted more quickly than intramuscularly injected antitoxin.
3. Very shortly after the intravenous injection of serum some of this antitoxin is excreted into the saliva through the salivary glands. From this moment the saliva is atoxic, so that henceforth no toxin can be absorbed from this saliva through the inflamed and damaged tonsils and wall of the pharynx. After the intramuscular injection of serum, it usually takes many hours, sometimes even days, for this antitoxin excretion through the salivary glands to get going. During this interval the saliva contains toxin which can be absorbed through the inflamed tonsils and wall of the pharynx.
4. Lesions of the heart (myocarditis and fatty degeneration) will very probably be less frequent among the intravenously injected patients than among those intramuscularly injected with antitoxin. These lesions will presumably also be less serious among the first group than among the second group of patients.
5. As a group, intravenously injected patients will very probably show fewer cases of paralysis than patients intramuscularly injected with serum.
6. Under comparable conditions, mortality among intravenously injected patients is likely to be lower than among intramuscularly injected patients.

There is no danger attached to the intravenous injection of serum, provided that certain precautions are taken. The skin test for hypersensitivity to the serum is especially important and should not be omitted.

For this test 0.2 ml of the serum, ten times diluted with saline, should be injected *i n t r a c u t a n e o u s l y* into the skin of the inside of the forearm. It is absolutely imperative that a wheal should form during this injection. If this is not the case, the in-

tracutaneous injection must be considered unsuccessful and will have to be repeated.

If no redness or swelling shows within an hour after the intracutaneous injection, the intravenous injection can safely be performed. Our experience with many hundreds of patients is that in this case there are never any serious reactions. Some hours after the intravenous injection of serum (enzymatically purified, radically despeciated horse serum) a few of the patients had a slight temperature, sometimes accompanied by a rigor. This type reaction proved to be a harmless concomitant.

If, however, the skin reaction is positive (redness and/or swelling), the serum must be injected intramuscularly and in very small portions at intervals of half an hour or an hour under the supervision of an experienced person who, if necessary, can administer adrenaline (0.1 – 1.0 ml adrenaline-HCl 1:1000 depending on the age) if the patient shows obvious allergic symptoms.

For the sake of completeness, mention may here be made of a publication by PARISH, LAURENT and MOYNIHAN (1957). These authors propose a hypersensitivity test in which 0.2 ml of undiluted serum is injected subcutaneously.

If there are no general reactions within 30 minutes, the total amount of serum can be injected either subcutaneously or intramuscularly without special precautions. This refers to the injection of a prophylactic dose of antitetanus serum (generally 1500 A.U. in 1 ml).

We are not disposed to recommend this prescription with regard to the therapeutic treatment of diphtheria patients. We believe that the risk of serious allergic symptoms is much greater after subcutaneous injection of 0.2 ml of undiluted antidiphtheria serum (4000 A.U. per ml, the protein content generally being higher than that of prophylactic antitetanus serum), than after the test-injection described by us.

The doses of serum which we have given in the last few years varied from 10,000 to 20,000 A.U. per patient, according to age. The results of the determination of antitoxin titres after the intravenous injection have convinced us that an additional intramuscular injection of diphtheria serum would be superfluous.

Provided that the precautions described above are observed, there is, in our opinion, not the slightest objection to the intravenous injection of serum in all cases where an injection may be required. Consequently all these patients should be

injected not intramuscularly but intravenously with diphtheria antitoxin. The intravenous injection of serum in patients suspected of diphtheria ought therefore to become the rule and should not be reserved for "serious" or toxic cases, as is still usually done at present.

Besides, it is by no means always possible to predict with certainty what course the disease will take. During a diphtheria epidemic there have repeatedly been cases which initially seemed mild but which within a few hours assumed a pronounced toxic character with a rapidly fatal outcome. Some of these patients would probably have been saved if they had been given an intravenous anti-diphtheria injection at the earliest opportunity.

It should be borne in mind that with patients suspected of diphtheria the first object of the serum therapy is the quickest and completest possible elimination of any further absorption of diphtheria toxin (e.g. from the saliva) and the binding of the toxin which is still circulating. The investigations here described will have shown that, besides the promptness of the serum injection, the route by which this serum is administered can also have a decisive effect. Not until this has been done will the treatment be directed to combating the damage already done to the organs (heart, kidneys, nervous system).

Summary.

The investigation described has clearly brought out the advantages of the intravenous injection of serum in patients suspected of diphtheria, as compared with the intramuscular method which is still commonly used. These may be summed up as follows.

The intravenously injected antitoxin immediately brings the serum of the patient "up to titre", whereas absorption of the intramuscularly injected antitoxin is slow. On an average this absorption is not complete until after about four days, and may in certain cases even take up to six days. Intravenously injected serum is not excreted more rapidly than intramuscularly injected antitoxin.

Very shortly after the intravenous injection of serum a small portion of this antitoxin is excreted through the salivary glands into the saliva, thus making this atoxic; consequently, from this moment no more toxin will be absorbed from this saliva through the inflamed and damaged tonsils and wall of the pharynx.

Evidence from animal experiments indicates that patients intravenously injected with diphtheria serum may be expected to show fewer lesions of the heart (myocarditis and fatty degeneration), fewer post-diphtheritic paralyses and a lower mortality than patients intramuscularly injected with serum.

With due observance of the necessary precautions – such as a skin test for hypersensitivity, which must be negative – diphtheria antitoxin should therefore be injected not intramuscularly but intravenously, whenever such an injection is required, also including mild cases of diphtheria.

A c k n o w l e d g e m e n t s.

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FLAGELLATION AND TAXONOMY OF *ACETOBACTER* AND *ACETOMONAS*

by

J. L. SHIMWELL

(Received March 17, 1958).

Until 1954 all motile acetic acid bacteria were believed to possess polar flagella. For this reason, they had been classified, in Bergey's Manual of Determinative Bacteriology, 6th Ed. (1948), in the family *Pseudomonadaceae*, which, by definition, excludes bacteria with flagella other than polar. Furthermore, the motile species described therein are listed as possessing a single polar flagellum.

In 1954, however, LEIFSON, as the result of the examination of 30 representative strains, claimed that no acetic acid bacterium had a single polar flagellum, but that two distinct and different types of flagellation were to be found within the group, these being correlated with an equally distinct difference in an important biochemical activity. Thus all motile species capable of oxidizing acetic acid to CO_2 and H_2O possessed peritrichous flagella; those incapable of so doing – FRATEUR's "suboxydans group" (1950) – possessed multitrichous polar flagella, and these were of such short wavelength that a flagella-stain alone sufficed for identification.

LEIFSON therefore pointed out that a division of the acetic acid bacteria into two genera was necessary. He suggested that the genus *Acetobacter* should be retained for the inclusion of peritrichously flagellated species, and non-motile acetate-oxidizing ones, but that, for the others, a new genus "*Acetomonas*" should be established, which "should include only polar multitrichous species and non-flagellated species of similar physiology".

Acetomonas, having polar flagella, could obviously remain in the family *Pseudomonadaceae*, but *Acetobacter* (as newly defined), equally obviously, must be removed therefrom.

EXPERIMENTAL.

Flagellation of *Acetobacter* amended Leifson.

In the course of postal discussion on the effect of LEIFSON's work on the taxonomy of *Acetobacter* the late Dr ROBERT S. BREED (private communications) expressed some scepticism about the peritrichous flagellation of the acetate-oxidizing species, as LEIFSON's published photomicrographs, although clearly showing the shape of the flagella, did not conclusively demonstrate the all-important point of attachment to the cell-body. Although able to assure Dr BREED that I had amply confirmed the doubted peritrichous flagellation microscopically, I was, at that time, unable to produce convincing photographs suitable for publication, for, as LEIFSON rightly says, the acetic acid bacteria are more difficult subjects for flagella-staining than most bacteria.

However, in view of the scientific, taxonomic, and technological importance of LEIFSON's work, it was felt that all doubts should be removed, and various flagella-stains were persevered with and modified. Eventually a modification of the method of FISHER and CONN (1943), in which 1% aqueous crystal violet replaced both 5% alcoholic basic fuchsin and carbol-fuchsin, yielded photographable results. It is submitted that Fig. 1. demonstrates peritrichous flagellation in *A. rancens* beyond dispute. Similar results have been obtained with other acetate-oxidizing strains.

The peritrichous flagellation of *Acetobacter* proper has been further confirmed in an interesting way. Although all the celluloseless mutants I myself had obtained from the non-motile cellulosic species *A. xylinum* had been similarly non-motile, Dr J. G. CARR, of Bristol University (private communication), has obtained a motile celluloseless mutant from a cider strain of the same species. I have flagella-stained this mutant, and it has peritrichous flagella, the microscopic picture presented being virtually identical with Fig. 1, and therefore not illustrated.

This has been subsequently confirmed by Dr CARR himself, using the same staining method. Biochemically the mutant corresponds to *A. mesoxydans* Frateur. This finding supports LEIFSON's proposal to include both non-motile, acetate-oxidizing species and peritrichously flagellated strains in the same genus *Acetobacter*.

LEIFSON found that the peritrichous flagella of *Acetobacter* strains were "quite orthodox" and tended to be few in number,

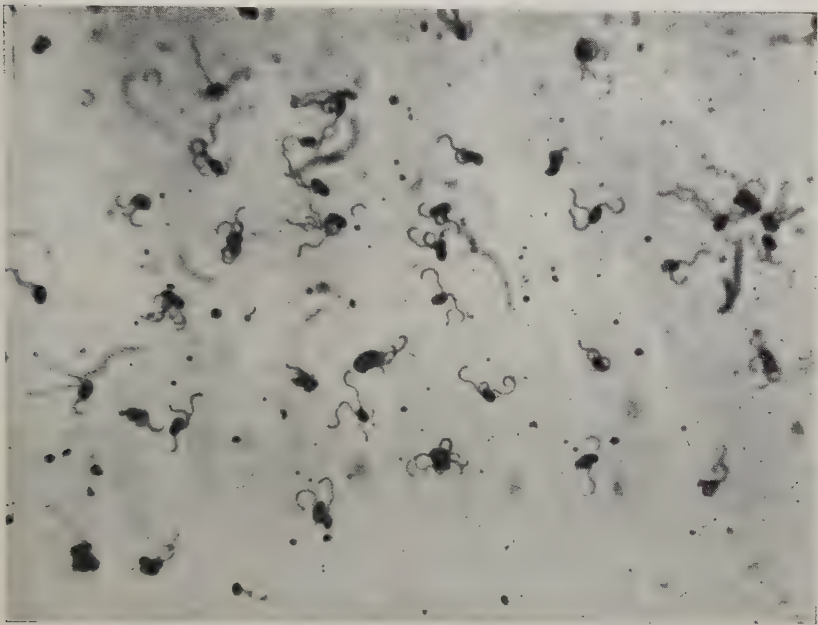


Fig. 1. *A. rancens*. Sparse peritrichous flagella of "long" wavelength. $\times 1500$.



Fig. 2. *A. aceti*. Numerous peritrichous flagella of short wavelength (1.4μ). $\times 1500$.

of very uneven arrangement, and with a wavelength averaging $2.9\ \mu$. Whilst I have confirmed this in most cases (e.g. Fig. 1) a strain of *A. aceti* (isolated by Dr CARR from cider) has been found to possess a peritrichous flagellation of a very different type. It will be seen from Fig. 2 that the flagella are numerous, and that their wavelength is of the ultra-short type ($1.4\ \mu$) considered by LEIFSON to be uniquely confined to the polar flagella of *Acetomonas*.

Flagellation of *Acetomonas* Leifson.

As previously mentioned, LEIFSON specifies that his new genus *Acetomonas* should exclude any motile strains unless these have "polar multitrichous" flagella. However, I found one strain of *A. melanogena* to possess only a single polar flagellum (Fig. 3). This strain was, both culturally and biochemically, typical *A. melanogena*, producing the deep red to black-water-soluble pigment characteristic of the species.

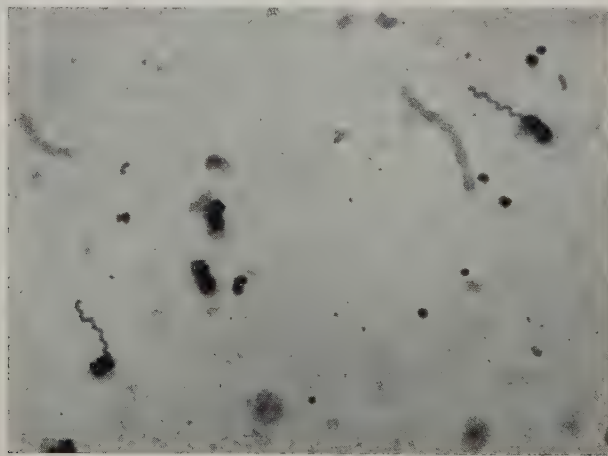


Fig. 3. *A. melanogenum*. Single polar flagellum of ultra-short wavelength ($0.9\ \mu$). $\times 1800$. (Note higher magnification).

On notifying Dr CARR (who had kindly supplied the culture) of this result, he was able to confirm it, and also found several other cider strains of this species with similar polar monotrichous flagellation. The motility was notably rectilinear, and somewhat sluggish.

The flagella of these strains became detached with exasperating

ease, despite every care, whilst preparing a film for staining. For this reason it was at first thought that the monotrichous appearance might be due to the detachment of all but one of probably multitrichous flagella from each cell. However, the examination of five separately prepared slides showed the number of flagella to be always either one or none. As several hundred of the former were observed, this seems to eliminate the detachment possibility.

As all the motile strains of *A. suboxydans* and *A. melanogena* examined by LEIFSON had polar multitrichous flagella it would seem that polar monotrichous strains may be of relatively rare occurrence; nevertheless they seem to exist.

It will be noted from Fig. 3, however, that LEIFSON's finding that the wavelength of the polar flagella is extremely short is fully confirmed. In fact the wavelength of the two flagella on the photograph will be seen to be only about $0.9\ \mu$, being even shorter than the average of $1.4\ \mu$ found by him.

Taxonomy.

(a) *Acetomonas*.

From the foregoing it would seem that LEIFSON's original diagnosis of *Acetomonas* gen. nov. is somewhat too narrow, and should be slightly amended. It is suggested that for "polar multitrichous species" etc. the words "species with one or more polar flagella" should be substituted. As previously mentioned, the genus obviously remains in *Pseudomonadaceae*.

(b) *Acetobacter*.

As this genus cannot remain in *Pseudomonadaceae* on account of its peritrichous flagella, the only question is in which family it should be placed. As these organisms show little evidence of phylogenetic relationship to those of any existing family, I suggest that *Acetobacteriaceae* should be revived, as in the 5th (1939) edition of Bergey's Manual. This will admittedly result in a family with only one genus, and that genus (owing to facile mutation) containing perhaps only one or two species (SHIMWELL, 1957), but one should not, perhaps, expect bacteria to fit comfortably into a botanical type of classification originally devised for higher organisms.

Summary.

LEIFSON's findings, that motile, acetate-oxidizing acetic acid

bacteria (*Acetobacter*) have peritrichous flagella, and that motile, non-acetate oxidizing ones (*Acetomonas*) have polar flagella, of notably short wavelength, are fully confirmed and photographically illustrated.

It is not confirmed, however, that the peritrichous flagella of *Acetobacter* are always of "orthodox" type with a wavelength of about $2.9\ \mu$, nor that they always tend to be few in number. In one strain of *A. aceti* they were numerous, and the wavelength was as short ($1.4\ \mu$) as that considered by LEIFSON to be uniquely confined to the polar flagella of *Acetomonas*.

Furthermore the polar flagella of the latter genus seem not always to be multitrichous, strains having been found with only a single polar flagellum.

Acknowledgements.

I wish to thank the Directors of British Vinegars Ltd., London, for permission to publish this paper, and Dr J. G. CARR, of Bristol University, for cultures and collaborative discussions.

Addendum.

Since the above paper was written, Dr EINAR LEIFSON (private communication) has examined the cultures of *A. aceti* and *A. melanogena* referred to, and has confirmed the numerous short-wave flagella of the former, and the presence of a single polar flagellum on many cells in the latter. He also found some multitrichous polar flagellated cells in the *A. melanogena* strain, however, and I have subsequently confirmed this.

Dr LEIFSON concurs with my broadening of his original diagnosis of *Acetomonas* to include strains with „one or more polar flagella”.

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ON THE ISOLATION, ECOLOGY AND TAXONOMY OF *SACCHAROMYCOPSIS GUTTULATA*

by

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(Received May 12, 1958).

The present limited knowledge of the yeast *Saccharomyopsis guttulata* (Robin) Schiønning is the result of sporadic studies since 1845. Undoubtedly, the main reason for the paucity of information on this interesting organism is, that it has resisted, until recently, all efforts to grow it in pure culture. It apparently requires a certain growth factor(s) not present in media generally used for the cultivation of yeasts. Due to the difficulty of culturing this yeast the data in the literature are based largely on observations of the yeast in its natural habitat and in enrichment cultures.

REMAK (1845) was the first to observe large cylindrical yeast-like cells in the stomach and intestinal contents of a rabbit and of certain other herbivores. He did not find it in carnivores, birds or reptiles. ROBIN (1853) confirmed the presence of this yeast in the intestinal tract of rabbits and of certain other herbivores. He named it *Cryptococcus guttulatus* on the basis of the very characteristic hyaline vacuoles in the cells. Terminal budding was observed and the cells occurred singly, in pairs or chains of three. The dimensions were $(6-8) \times (15-20)\mu$. WINTER (1884) termed the yeast *Saccharomyces guttulatus*, although sporulation had not been observed at the time. BUSCALIONI (1896) and BUSCALIONI and CASAGRANDE (1898) were the first to obtain sporulation of the yeast by alternately wetting and drying fecal matter of rabbits. One to four ascospores were observed. The finding of sporulation justified acceptance by the last two authors of the generic designation *Saccharomyces*. Attempts to germinate the spores failed. Some growth was claimed to occur on an agar medium containing glycerol and glucose to which tartaric acid was added to establish a low pH. Inversion of sucrose

and formation of some alcohol from glucose by cells enriched in an acidic medium was also demonstrated. A very thorough study was made by WILHELMI (1898), who confirmed the occurrence of the yeast in rabbits. He did not observe it in a large number of other herbivorous mammals included in his study. All experiments designed to culture *S. guttulatus* on nutrient media generally suitable for the growth of yeasts were fruitless. Enrichment cultures in acidified hay extract plus 10 per cent glucose produced good growth at 37°C. when inoculated with stomach contents. The culture could be transferred to a liquid medium of the same composition, but purification by plating was not accomplished due to lack of growth on solid medium. WILHELMI confirmed sporulation and noted that it occurred at low temperatures (*e.g.* 14°C.) in contrast to growth of the vegetative cells which was obligatory at 37°C. He observed germination of the double walled spores at 39°C. First the spores swelled, the outer spore wall ruptured and a new yeast cell protruded containing its own less refractive wall. Diploidization during germination was not observed. In addition WILHELMI noted that the vegetative cells were extremely short lived. A maximum of about three days was indicated. SCHIÖNNING (1903) established a new genus *Saccharomycopsis*, to accomodate a newly isolated yeast with a double spore membrane. The species was designated *S. capsularis* because of the presence of an exosporium during germination of the spores. On the basis of the germination studies of WILHELMI (1898), SCHIÖNNING (1903) transferred *Saccharomyces guttulatus* to the new genus *Saccharomycopsis*, retaining the species name which should have been modified to *S. guttulata*. GUILLIERMOND (1909) on the basis of comparative studies of *S. capsularis* Schiönnig and *Endomyces fibuliger* Lindner came to the conclusion that they are closely related. He considered both to belong to the genus *Endomyces* Reess. GUILLIERMOND therefore renamed SCHIÖNNING's species *Endomyces capsularis*, thus removing the type species from the genus *Saccharomycopsis*. Subsequently STELLING-DEKKER (1931) placed *Endomyces capsularis* in the genus *Endomycopsis* retaining the species name. STELLING-DEKKER retained provisionally *S. guttulata* as the only species in the genus *Saccharomycopsis*.

COCHET (1940) studied extensively the fungal flora of the intestines of certain rodents. She observed cells of *S. guttulata* in the intestines of rabbits, hares and rats. She was unable, however, to obtain pure cultures. LODDER and KREGER-VAN RIJ (1952) studied the organism

in enrichment cultures of malt extract at pH 2.5 and at 37°C., using stomach contents or fecal pellets of rabbits as inoculum. Transfers of the enrichment culture to liquid or solid media of the same composition did not produce growth. After our own work, to be described in this paper, had been completed, PARLE (1956) reported for the first time a method to obtain pure cultures of *S. guttulata*. He prepared a medium consisting of an extract of rabbit stomach contents, yeast extract (Difco) and glucose. The pH was adjusted to 3.5. Pure cultures were obtained by serial transfers in the above medium incubated at 37°C. Colonies did not develop in pour plates or on the surface of agar to which the above medium was added. The fermentation of glucose and sucrose was confirmed. Asci with one or two spores were found on heavily inoculated Gorodkova slants and on turnip wedges after two weeks of incubation at 18°C. ROLLE and MEHNERT (1957) observed *S. guttulata* in the stomach of healthy rabbits and chinchillas. The yeast was not present in the stomach of healthy rabbits which were milk fed, but only in those fed with hay or straw. PARLE (1957) surveyed the yeast flora in the alimentary tract of cows, pigs, opossums, rabbits, sheep, monkeys, cats, dogs, hedgehogs, mice, guinea pigs and rats. Microscopic examination showed that *S. guttulata* was present in all of the 30 rabbits sampled. The yeast was not found in any of the other animals studied.

The purpose of the present paper is to describe a simple reproducible method of obtaining and propagating pure cultures of *S. guttulata*. The taxonomic position and the ecology of *S. guttulata* will be discussed. The nutritional requirements of the yeast will be described in a separate communication (SHIFRINE and PHAFF, 1959).

ISOLATION OF PURE CULTURES.

Microscopic examination of suspensions of fecal matter readily revealed the presence of the large cells of *S. guttulata*. Fecal pellets of rabbits raised entirely on dehydrated food usually did not contain the yeast in contrast to animals which were, at least partly, fed on fresh vegetables. The soft night feces appeared to contain more yeast cells than the dry day pellets.

Several grams of fecal matter were suspended in a flask (capacity 125 ml) containing 50 ml of yeast autolysate (10 per cent v/v) and 2 per cent glucose. The yeast autolysate was prepared according

to the procedure described by BOUTHILET *et al.* (1949). One per cent yeast extract (Difco) was not satisfactory, since it did not support continued growth on serial transfer of an enrichment culture. One per cent yeast autolysate (Albimi), however, supported good growth. The medium was adjusted with 2 N sulfuric or hydrochloric acid to a pH of 2.0 to 2.5 to prevent bacterial growth. After 2 to 3 days at 37°C. an enrichment culture of *S. guttulata* was available and one ml of the culture was transferred to 50 ml of a medium of the same composition. After 3 days at 37°C. 0.1 ml of the culture was spread with a bent glass rod on a plate containing yeast autolysate-glucose-agar, pH 5.5—6.0. In order to prevent the plates from drying out, they were placed in a dessicator, the bottom compartment of which was filled with water to maintain a high humidity. A high humidity appears to be essential for the growth of *S. guttulata* since growth on agar plates could not be obtained otherwise. Colonies of *S. guttulata* were observed after 2 to 3 days. The absence of bacterial colonies on the non-acidified medium indicated that the culture was free of bacterial contamination. After microscopic inspection a colony was transferred to a tube of yeast autolysate-glucose.

CYTOLOGY.

When grown either in liquid or on solid media the cytoplasmic changes of the cells, with time, are very striking. In cells 24 hours old the cytoplasm is mostly hyaline and it contains very few if any vacuoles or granules. After 48 hours the cytoplasm becomes somewhat granular and it contains one or more vacuoles, usually two. After 72 hours or longer the cytoplasm becomes more granular. Since buds have continued to form the culture medium at this time also contains healthy appearing clusters. In addition to vacuolation and granulation some cells rupture and their cytoplasmic contents flow out. The break in the cell wall seems to occur always at one of the long sides of the cylindrical cells and hardly ever at the pole. The culture medium at this stage contains numerous granules. The majority of these granules stain brown with Lugol solution, which is indicative of their glycogen content. Other granules stain dark with Sudan black B. These probably are lipid globules.

LIFE CYCLE OF *S. guttulata*.

Conditions for sporulation.

Observation of sporulation with subsequent germination is essential for the study of the sexual life cycle of a yeast.

In order to find the optimal conditions under which ascospores are formed a variety of sporulation media were used. After heavy inoculation with the sediment of a liquid culture the media were incubated at 18°, room temperature (22°—25°), 30°, 37° and at ambient outdoor temperature (*ca.* 6° to 25°C.). The latter was chosen to allow normal temperature fluctuation to approximate similar variations in voided rabbit feces. Microscopic observations were made daily. The greatest number of spores occurred on yeast extract-malt extract-peptone-glucose-agar (YM, Difco) after 5 days of incubation. Sporulation was observed only at room temperature and at 18°C. Most spores were observed at the latter temperature.

It is of interest to note that although no growth or multiplication occurs at 18°C., some metabolic processes are operative culminating in the formation of ascospores.

Ascospores are oval to cylindrical $(0.9\text{--}1.6) \times (1.0\text{--}2.8)\mu$; rarely a round spore was observed. The number of ascospores per ascus is one to four (See Fig. 1). A five spored ascus was seen once. The number of the different spored asci varies in different preparations. However, asci with two spores are always in the majority and asci with one spore are least common. Characteristically ascospores lie free in the ascus. With time the spores swell and sometimes fill up the ascus. This occurs mainly with asci containing 3 or 4 spores. Asci do not rupture when mature. Conjugation of vegetative cells does not precede ascus formation.

Nuclear behavior.

The behavior of the nucleus was followed from the diploid vegetative cell through the spore forming stage to the individual ascospore.

The nuclei were stained by the following procedure: A suspension of vegetative cells was placed on a slide and allowed to dry at room temperature. After fixation in Schaudin's fixative for 20 minutes, the preparation was hydrolyzed with 1N perchloric acid at 4°C. for 72 to 84 hours. The cells were washed with distilled water and

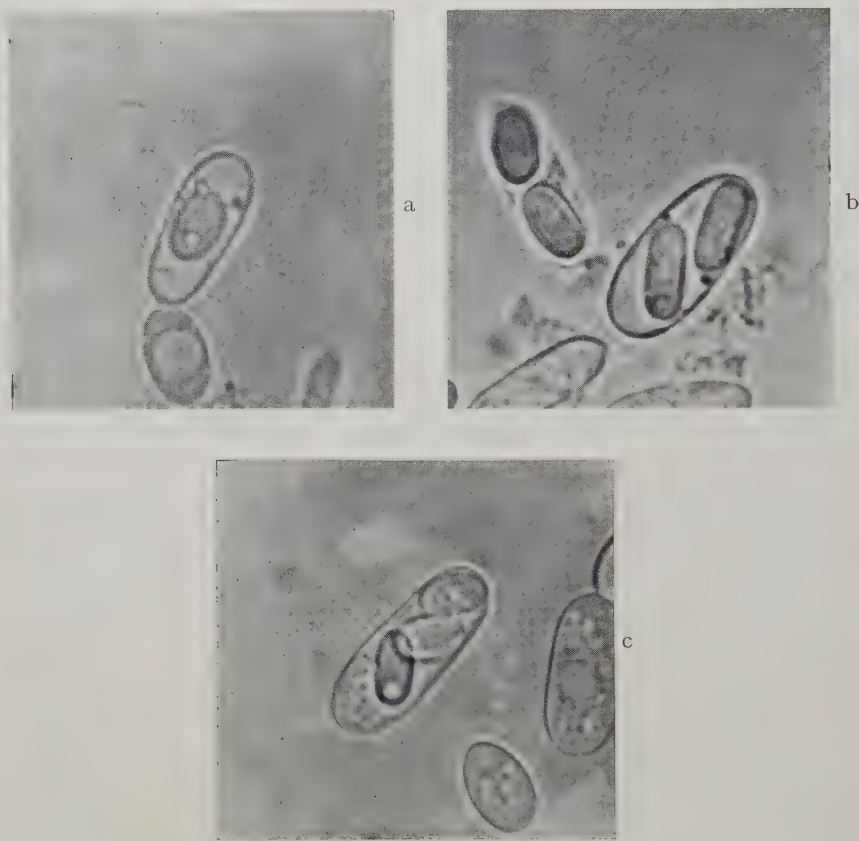


Fig. 1. a, b, c. Asci of *S. guttulata* containing one, two and four spores.

flooded with Azure A¹) (0.1%) for one minute. The slide then was washed with distilled water and dried.

Vegetative cells are uninucleate (Fig. 2a). In order to determine the nuclear picture during meiosis cells were removed from the sporulation medium after 3 and 4 days and stained. The majority of the cells undergoing meiosis manifested two nuclei; in some there were three or four nuclei (Fig. 2b, c, d). It is difficult to determine whether, in the case of asci with less than 4 ascospores, first 4 nuclei are formed followed by degeneration of one or more nuclei, or whether less than four are formed originally.

¹) Azure A, National Aniline Division, Allied Chemical and Dye Corp. N. Y. Certif. No. N Az 15.

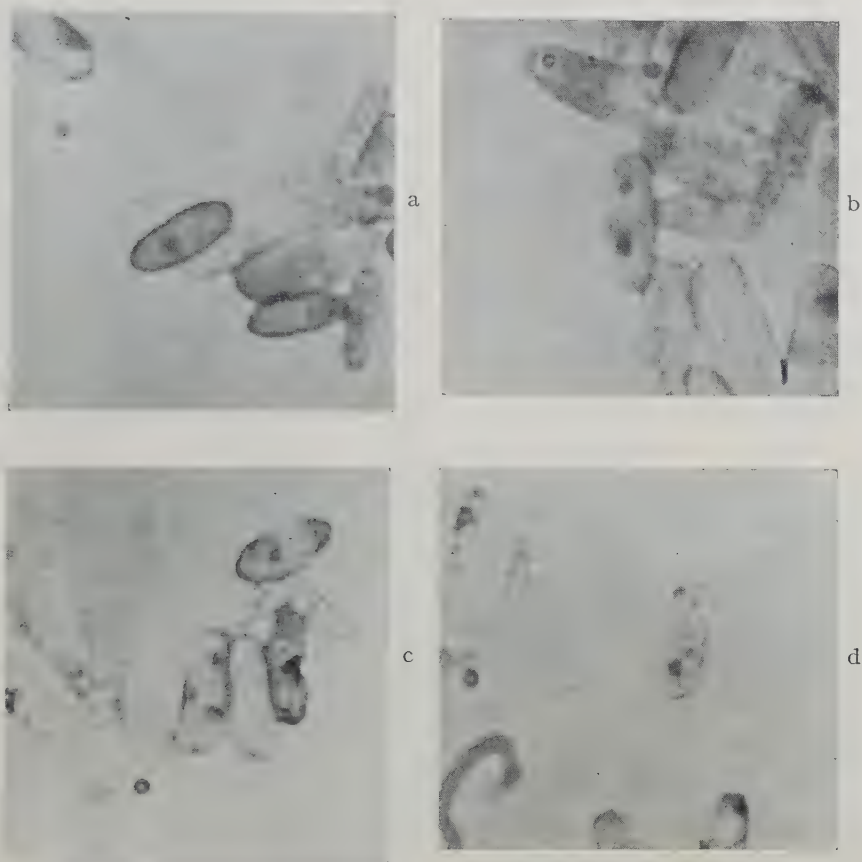


Fig. 2. Stained nuclei of *S. guttulata*. (a) uninucleate vegetative cell; (b) (c) and (d) cells undergoing meiosis with two, three and four nuclei, respectively.

The nuclear behavior in the different stages of the life cycle of *S. guttulata* indicates that the vegetative cells are diploid (or perhaps of a higher ploidy), and that the spores are haploid (or of a correspondingly higher ploidy).

Germination of ascospores.

Preliminary studies showed that it is essential to grow the yeast at pH 6.0 in order to obtain a reasonable percentage of viable ascospores. Cells 48 hours old were placed on the sporulation medium and incubated at 18°C. After 5, 6, or 7 days the culture from YM agar, containing cells and asci, was placed in a sonic oscillator in

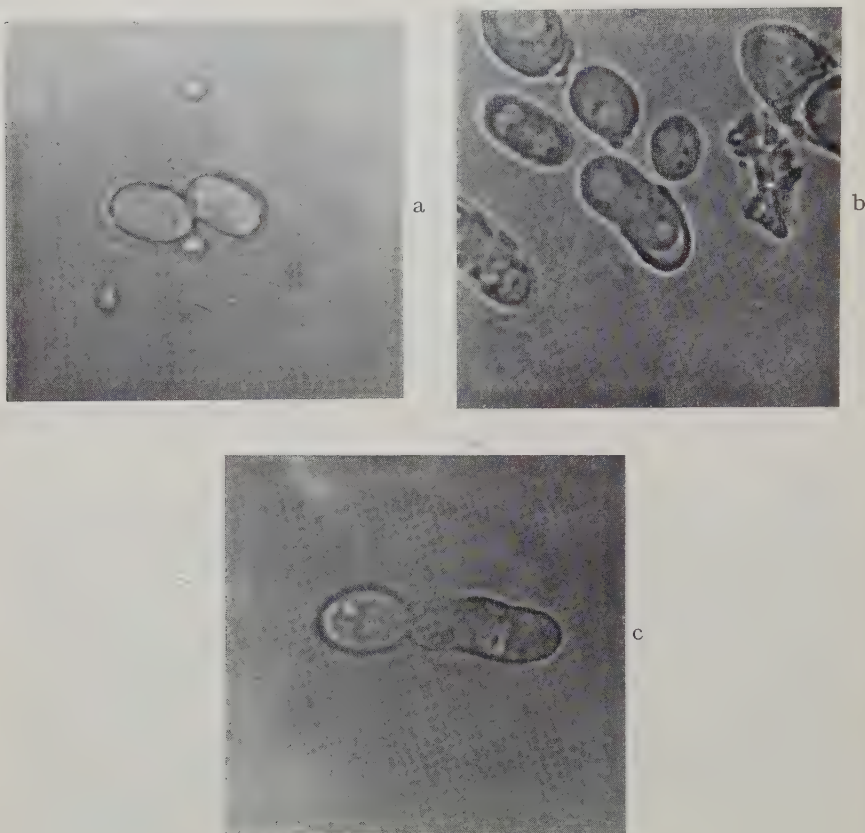


Fig. 3. Germination of spores. (a) conjugation between two spores; (b) germination of a single spore showing the exosporium; (c) same as (b) showing the first bud formed.

order to rupture most of the vegetative cells. A certain percentage of the asci also breaks. The spores are quite resistant to sonication. The material was then inoculated into yeast autolysate-Proteose Peptone-glucose (pH 4.5 or 6.0) and incubated at 37°C. One per cent Proteose Peptone (Difco) was added to the usual medium, since it was found to improve growth (SHIFRINE and PHAFF, 1959).

Spores were observed to germinate after conjugation (Fig. 3a), but also without conjugation (Fig. 3 b, c). It is not known whether the result of germination of a single spore is a haploid or a diploid culture. In some cases a single ascospore germinated apparently without conjugation when two spores were present inside the ascus

(Fig. 4a, b). The resulting blastospores from a single ascospore were of smaller size than the buds resulting from ascospores which had conjugated. If the small cells are assumed to be haploid, the smaller size may be explained on the basis of results obtained with *S. cerevisiae* by MUNDKUR (1953), who showed that the volume of cells is directly proportional to their ploidy.

The writers' findings show that the germination of a spore of *S. guttulata* is a unique process characteristic of this yeast. Prior to germination spores swell and fill up the ascus. When a germination tube is formed the exosporium ruptures and remains around the base of the spore (Fig. 3b, c). The germination tube then proceeds to bud (*cf.* Fig. 3c, 4b) and forms a chain of cells. This completes the sexual cycle of *S. guttulata*.

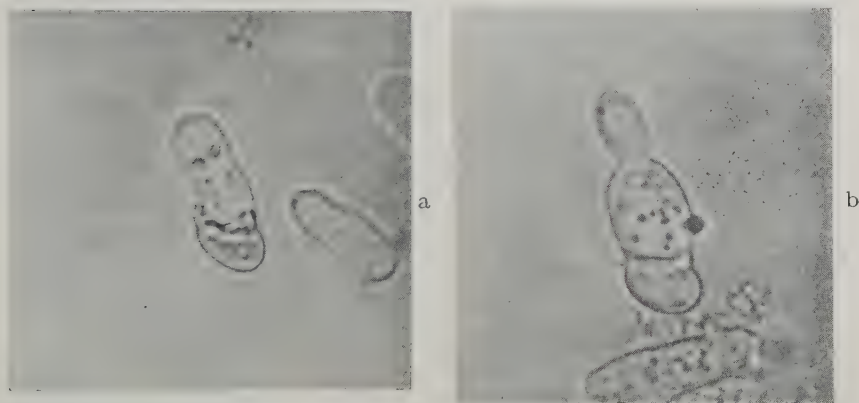


Fig. 4. Germination of spores. (a) one of a pair of ascospores is forming a germination tube; (b) same as (a) showing the first bud formed.

Unfortunately only a small part of the total number of ascospores germinate. Asci could still be found in a medium inoculated with sporulating cells and grown for one week at 37°C. Moreover, at least 75 single spores and single asci were isolated with the aid of a micromanipulator and placed in the nutrient medium, but none germinated.

Since the majority of the ascospores, in mass cultures, conjugate prior to germination the original ploidy of the vegetative cells is reestablished. The nuclear picture of a culture resulting from the germination of a single spore is not known. It is proposed that the

scheme shown in Figure 5 represents the life cycle of *Saccharomyopsis guttulata*.

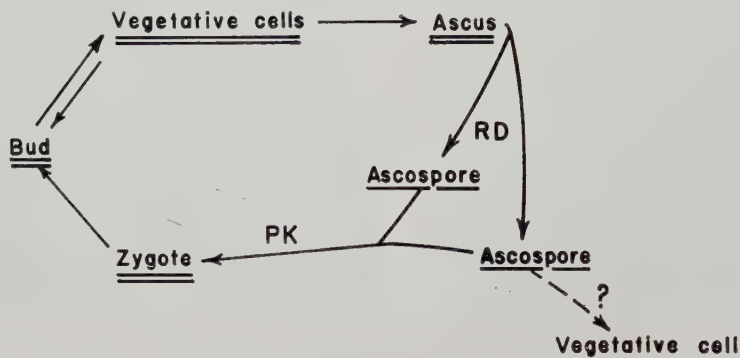


Fig. 5. Proposed life cycle of *S. guttulata*. A double line represents a diploid cell and a single line represents the haploid condition. RD and PK refer to reduction division and plasmogamy and karyogamy, respectively.

LONGEVITY AND MAINTENANCE OF CULTURES OF *S. guttulata*.

Previous investigators who worked with *S. guttulata* (WILHELM, 1898; PARLE, 1956) indicated that this yeast is short lived, *i.e.* 3 to 7 days. A study of the longevity of cells of *S. guttulata* at different temperatures was undertaken in the hope of finding a way to maintain a viable culture for a more prolonged period of time.

Cells were grown for 48 hours at 37°C. in 125 ml Erlenmeyer flasks containing 25 ml of yeast autolysate-glucose. The flasks were stored at 5°, 25° and 35°C. Samples of 0.1 ml were withdrawn every three days, inoculated into tubes containing 5 ml of fresh medium of the same composition, and incubated at 37°C. Growth in the fresh medium was used as a criterion of viability of the test cultures. The cultures incubated at 25°, 37° and 5°C. remained viable for 6, 12 and 61 days, respectively.

A similar experiment was done with a sporulating culture. A spore preparation was harvested from YM agar after 5 days of incubation at 18°C. The culture was centrifuged under aseptic conditions, the supernate was discarded, and the cells were resuspended in yeast autolysate-proteose peptone-glucose, pH 4.5. The preparation was stored in a refrigerator (*ca.* 5°C.). Aliquots of 0.1 ml were withdrawn weekly and inoculated into a fresh medium of the same composition, 5 ml per tube, and incubated at 37°C. Spores retained their viability for 6 months, at which time the culture

became contaminated with bacteria. Thus, maintaining a sporulating culture in a refrigerator for six months, affords a relatively simple way of keeping a viable culture of *S. guttulata*. WICKERHAM and BURTON (personal communication) have found that a lyophilized culture of vegetative cells remained viable for at least 18 months.

ECOLOGY.

For a better understanding of the relationship of *S. guttulata* to the rabbit, a brief review of coprophagy is useful. Coprophagy is practiced occasionally by most rodents, but in rabbits it is a normal habit. MOROT (1882) observed that rabbits excrete two types of feces. A dry hard pellet is voided chiefly during the day, while the feces voided at night is soft, lighter in color, and covered with a mucous membrane. Soft feces, which constitute about 1/3 of the total fecal matter, is consumed directly from the anus by the rabbits. Coprophagy is not only a normal physiological process in rabbits grown in cages (EDEN, 1940), but this process also seems to occur in wild rabbits under natural conditions according to observations made by SOUTHERN (1940). The habit of coprophagy benefits the rabbit by increasing the utilization of dietary nutrients (THACKER and BRANDT, 1955) and by enhancing the vitamin nutrition (KULWICK *et al.*, 1953).

Three observations should be kept in mind for the following discussion of the life cycle of *S. guttulata* in nature. First, cells of *S. guttulata* can be induced to sporulate by alternately wetting and drying fecal matter at a temperature of about 18°C. This method probably simulates the conditions under which ascospores are formed in nature. Second, WILHELM (1898) and the authors have observed germination when ascospores were inoculated into a nutrient medium incubated at 37°C. Third, *S. guttulata* grows well in media with a pH range between 2 and 6.5 (optimum pH 4.5), but at higher pH values growth is very slow (SHIFRINE and PHAFF 1959).

There may be a relation between the highly specific association of *S. guttulata* with rabbits and the fact that rabbits are the only rodents known to have the habit of coprophagy. However, an explanation for the specific habitat of *S. guttulata* in this rodent is not available. Since no other animal (with the possible exception of chinchillas) harbors *S. guttulata*, most probably this yeast cannot

become established in their gastro-intestinal tract. WILHELM (1898) was unsuccessful in his experiments to introduce this yeast into the intestinal tract of herbivorous animals other than rabbits.

How does *S. guttulata* maintain itself in the gastro-intestinal tract of rabbits? When the stomach contents are examined microscopically, actively budding cells of *S. guttulata* can usually be observed. PARLE (1956) demonstrated that an extract of the stomach contents of rabbits contains a growth factor required by *S. guttulata*. It is not known, however, whether this growth factor originates in the vegetative diet, in the soft night feces, in the gastric secretions or by a combination of these factors. Thus, coprophagy may affect the yeast flora in the stomach by (1) reinoculating the stomach with yeast cells present in the soft feces and (2) by supplying possible growth factors. Prevention of coprophagy, by stanchioning a rabbit, would be necessary to determine whether or not *S. guttulata* can maintain itself in the stomach under such conditions.

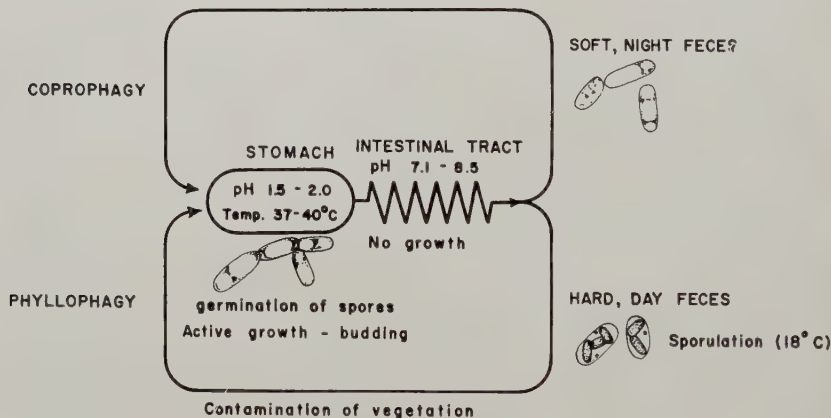


Fig. 6. Cycle of *S. guttulata* in nature. See text for details.

On the basis of available evidence the following cycle of *S. guttulata* in nature is proposed (Fig. 6): The vegetative cells propagate by budding in the stomach of a rabbit. On passage through the intestinal tract growth stops or continues to a very limited extent due to the high pH of the environment; the rabbit then excretes its feces containing the yeast cells. Upon ingestion of the night feces the yeast is brought back into the stomach of the rabbit and the cycle is completed.

On occasions when not all of the soft feces is ingested, or when hard feces is excreted, some vegetative diploid cells permanently leave the alimentary canal. If the temperature and moisture conditions are suitable, the cells sporulate. The asci, within the feces, are distributed by animals, insects, wind and other vectors and contaminate vegetation upon which, eventually, a rabbit might feed. In the stomach the ascospores germinate and the vegetative cycle is reestablished.

The fact that vegetative cells can produce ascospores when they are removed from the rabbit is of prime importance. The formation of spores affords a way for the yeast to survive for long periods of time outside the animal body, since the spores are more resistant to adverse conditions than are vegetative cells.

It was observed by several investigators (BUSCALIONI, 1896; WILHELM, 1898; ROLLE and MEHNERT, 1957) that suckling rabbits do not contain cells of *S. guttulata*. The yeast becomes established only after the rabbits are weaned. These authors attributed the first appearance of the yeast to the vegetative nutrition that started when the rabbits were weaned. In view of the fact that rabbits are coprophagous, a more plausible explanation might be the ingestion of soft feces of older rabbits by the weanlings.

TAXONOMIC CONSIDERATIONS.

SCHJÖNNING's description (1903) of the genus *Saccharomycopsis* reads as follows: "Budding yeast cells with endospores. The spore has two membranes and germinates by budding." The observation of a double membrane of the spore originally made by WILHELM (1898) has not been confirmed by any of the other investigators who studied *S. guttulata*. This limited definition of the genus, especially if the character of a double spore membrane is disregarded, does not differentiate clearly between the genera *Saccharomycopsis* Schjønning and *Saccharomyces* (Meyen) Reess.

Based on our present knowledge of the properties of *S. guttulata* reported in this paper and elsewhere (SHIFRINE and PHAFF, 1959) a better basis for comparison between the two genera is now available. Properties typical of *S. guttulata* are: (1) Certain amino acids are required for growth (SHIFRINE and PHAFF, 1958). (2) Growth occurs only between 35° and 40°C. (3) Ascospores germinate with the simultaneous shedding of an outer membrane. (4) The yeast

has a highly specific habitat. Normally it is only found in the gastro-intestinal tract of rabbits. (5) The fermentative ability of *S. guttulata* is considerably weaker than that of species of the genus *Saccharomyces*. (7) The cells of *S. guttulata*, especially when grown on solid medium, lyse after 7 or more days and therefore are short lived. After lysis only ghosts, granules and cell wall fragments can be observed. (8) The cell wall composition of *S. guttulata* differs appreciably from that of *S. cerevisiae* (SHIFRINE and PHAFF, 1958).

These characteristics sharply delineate the genus *Saccharomycopsis* from the genus *Saccharomyces*. They seem to the authors of sufficient importance to warrant acceptance of *Saccharomycopsis* as a valid genus.

Since the original description of the genus *Saccharomycopsis* by SCHIÖNNING (1903) is incomplete a supplementary description follows.

GENUS *Saccharomycopsis* SCHIÖNNING.

a. Diagnosis of the genus.

Cells long-oval to cylindrical, single, in pairs or in chains. Vegetative reproduction by budding at the poles or on a broad shoulder. Primitive pseudomycelium is formed in liquid media. Ascospores are oval to cylindrical, one to four per ascus. No conjugation of cells prior to sporulation. Upon germination an exosporium is evident. In liquid media a sediment is formed. Fermentation of sugars is weak. Amino acids are required for growth. Growth occurs only between 35° and 40°C.

b. The type species of the genus.

Saccharomycopsis guttulata (Robin) Schiönnig, the only species in this genus, is the type species.

c. Historical survey of the generic name ¹⁾.

The genus was established by SCHIÖNNING in 1903. He gave the following diagnosis: Budding yeast cells with endospores. The spore has two membranes and germinates by budding.

d. Synonyms:

Cryptococcus guttulatus Robin (1853)

Saccharomyces guttulatus (Robin) Winter (1884)

¹⁾ cf. Review of the literature.

e. Standard description of Saccharomycopsis guttulata (Robin) Schiöningg.

Growth in liquid medium (yeast autolysate (10% v/v)-proteose peptone (1%)-glucose (2%), pH 4.5): Cells are long-oval to cylindrical. A bud usually appears at one pole, it elongates with time and becomes cylindrical. After two days branched chains can be observed (Fig. 7). In many cases the original mother cell appears dead by the time the chain is formed, as judged by complete granulation of its protoplasm. After 48 hours the range of cell dimensions is $(4.4-6.1) \times (14-21)\mu$. No ring or pellicle is formed, even after an extended period of incubation. The cells have a tendency to grow on the glass wall of the tube, about one cm below the surface of the liquid. In older cultures the cells form a loose sediment.

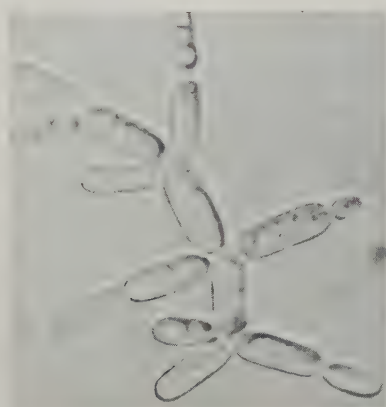


Fig. 7. Branched chains of vegetative cells growing in a liquid medium.

Growth on solid medium. When a plate of yeast autolysate-proteose peptone-glucose-agar (pH 4.5) is inoculated with cells of *S. guttulata* colonies appear after 48 hours. Cells are oval to cylindrical $(4.4-7.9) \times (8.8-19.5)\mu$, mainly single or in pairs.

Sporulation. Spores are formed on YM agar (Difco) after 5 days at 18°C. No conjugation immediately preceding ascospore formation. Consequently vegetative cells are presumed to be diploid. Spores are oval to cylindrical $(0.9-1.6) \times (1.0-2.8)\mu$. Usually two spores per ascus, sometimes one, three or four and rarely five. Some asci may rupture. Ascospores germinate directly or after conjugation. Germination tubes are formed with a simul-

taneous shedding of an outer spore membrane (exosporium). Germination is followed by formation of buds.

Fermentation. There is a weak fermentation of glucose, sucrose and raffinose. Galactose, maltose, lactose and melibiose are not fermented.

Assimilation (determined in 10% v/v yeast autolysate containing 2% of the carbon source). Glucose, sucrose, raffinose and citrate (weakly) are assimilated. Galactose, L-sorbose, maltose, cellobiose, trehalose, lactose, melibiose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, glycerol, i-erythritol, adonitol, dulcitol, D-mannitol, D-sorbitol, alpha-methyl-D-glucoside, salicin, Na-gluconate, Ca-2-ketogluconate, K-5-ketogluconate, Na-lactate, Na-succinate and i-inositol are not assimilated.

Assimilation of nitrate. This property could not be determined due to the complex nature of the medium required for growth.

Temperature range for growth. Growth occurs only between 35° and 40°C.

S u m m a r y.

A simple procedure is described for the isolation of pure cultures of *Saccharomycopsis guttulata* from rabbit feces. Sporulation occurs best on YM agar at 18°C., whereas vegetative growth takes place only between 35° and 40°C. On solid media growth occurs only in an atmosphere saturated with water vapor. Vegetative cells are diploid and uninucleate. Upon reduction division two to four nuclei could be observed after staining with Azure A, resulting in asci with one to four spores. Viability of the spores is low. In mass cultures, spores in some asci germinated after conjugation in pairs and in some, single spores germinated. During germination an exosporium is evident. A tentative life cycle has been proposed. *S. guttulata* is short lived at room temperature or above. However, at 5°C. a vegetative culture remains viable for about 2 months and a sporulating culture for at least six months. The ecology and natural cycle of *S. guttulata* have been described and related to the habit of coprophagy in rabbits. A complete description of *S. guttulata* is given, including its morphological and physiological properties. The genus *Saccharomycopsis* is considered to be valid.

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TORULOPSIS PSEUDAERIA NOV. SPEC., A NEW YEAST FROM SOIL

by

J. ZSOLT

(Received February 28, 1958).

A yeast was isolated from the soil of a vineyard of the Hungarian wine district Badacsony (beside Lake Balaton). The strain was investigated by the standard procedures of LODDER and KREGER-VAN RIJ (1952) and KUDRIAWTZEW (1954). Some other characteristics were also noted. The strain appeared to be a representative of the imperfect genus *Torulopsis* but was not identical with any species so far described. For this reason it is described as a new species: *Torulopsis pseudaria*.

DESCRIPTION.

Growth in malt extract: After 3 days at 25°C., the cells are globular, 5–8 μ in diameter, single or in typical "torulopsis" groups (Fig. 1). After 1 month a sediment and a ring is formed, whitish islets are also present.

Growth on malt agar: After 3 days at 25°C. the same globular cells as in malt extract were observed. But at the same time there appeared also lemon shaped cells. The cell wall of the globular cells was thicker than that of the lemon shaped ones (Fig. 2). The plasma of the globular cells was yellowish and a large fat globule was observed in each of these cells. After 1 month at 20°C., the streak culture was cream colored, flat, smooth.

Growth on yeast extract agar (1 g (NH₄)₂SO₄, 0.2 g KH₂PO₄, 0.2 g MgSO₄·7H₂O, 10 g sugar, extract of 10 g baker's yeast, 20 g agar in 1 liter tap water): The microscopic appearance was the same as on malt extract agar. The ratio of the numbers of the globular and the lemon shaped cells was, however, somewhat

variable according to the sugar. After 1 month the streak cultures are whitish and strongly wrinkled.

Growth on synthetic agar: The microscopic and macroscopic appearance was identical with that on yeast extract agar. Rapid growth was observed without any vitamins.

Dalmau plate cultures: No pseudomycelium was formed (Fig. 3).

Sporulation: No sporulation was observed on Na-acetate agar.

Fermentation: No fermentation was observed.

Assimilation of different carbon sources: glucose +, galactose +, sucrose +, maltose +, lactose +, melibiose +, trehalose +, raffinose +, dextrine +, starch —, inulin —, arabinose +, xylose +, ethanol —, glycerol —, mannitol +, sorbitol +, dulcitol +, acetic acid +, lactic acid —, succinic acid —, fumaric acid —, malic acid —, tartaric acid —, citric acid +, gluconic acid +.

Assimilation of potassium nitrate: Positive.

"Starch" formation: Absent.

Carotenoid pigments: Absent.

Esculin splitting: Very weak.

DISCUSSION.

The above mentioned characteristics show that the strain would be a representative of the genus *Torulopsis* Berlese in the system of LODDER and KREGER-VAN RIJ. The strain as such — owing to its biochemical properties — could be determined as *Torulopsis aeria* (Saito) Lodder. However, the lemon shaped cells on malt agar, a morphological characteristic, which is valued very highly by LODDER and KREGER-VAN RIJ, indicate that the strain is different from SAITO's species. Lemon shaped cells are not mentioned in LODDER and KREGER-VAN RIJ's description based on the authentic type strain. According to their observations the cells of *Torulopsis aeria* are "almost round".

The lemon shaped cells of our strain differ from those of *Kloeckera*. The *Kloeckera* cells are reproduced by bipolar budding. The lemon shaped cells of *Torulopsis pseudaria* are rather monopolar. The one end is regularly more round than the other and budding occurs almost on the rounded end. The base of the new bud is pointed

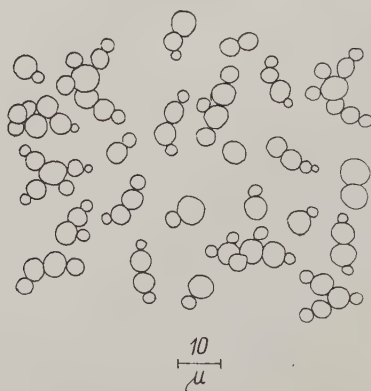


Fig. 1. *Torulopsis pseudaea*. After 3 days in malt extract.

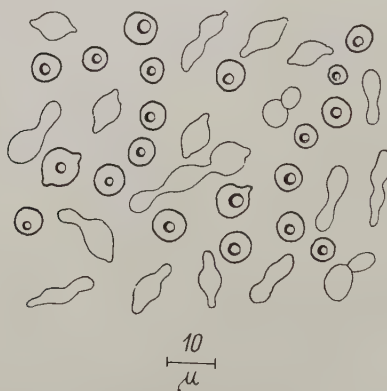


Fig. 2. *Torulopsis pseudaea*. After 3 days on malt agar.

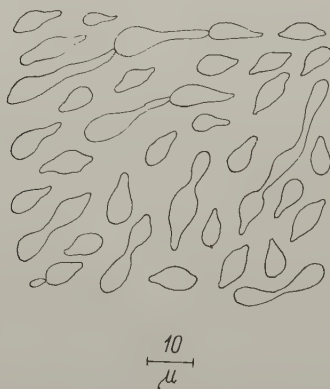


Fig. 3. *Torulopsis pseudaea*. Dalmau plate culture, potato agar.

again. This phenomenon may be very well observed on the Dalmau plate cultures (Fig. 3).

This morphological characteristic was the basis for the separation of the strain as a new species.

In addition to this the globular cells of *Torulopsis aerea* are 4–6 μ in diameter, while the cells of our strain are 5–8 μ .

SAITO's description in LODDER and KREGER-VAN RIJ (1952) indicates that *Torulopsis aerea* assimilates sugars weakly, lactose assimilation is uncertain. LODDER and KREGER-VAN RIJ found weak lactose assimilation. In our experiments with the new strain, vigorous growth was observed with all the sugars investigated. These characteristics confirm our view as to the separating of our strain from *Torulopsis aerea*.

In this study more characteristics are employed than it is done by LODDER and KREGER-VAN RIJ. Also KUDRIAWTSEW (1954), WICKERHAM (1951), PHAFF and KNAPP (1956) and BARNETT (1957) are of the opinion that this will undoubtedly prove necessary for the selecting of really essential characteristics for the classification of the yeasts.

The classification of our strain in the genus *Torulopsis*, however, is only provisional, as the whole of the Fungi imperfecti is in fact provisional. The thorough and elaborate work of the Dutch School created the possibility of research for new forms and in the later years considerable development took place in this field, more especially bearing on yeasts from soils (CAPRIOTTI 1955), VAN DER WALT (1956, 1957), VAN DER WALT and TSCHESCHNER (1957), ZSOLT (1957) and unpublished results from the present author). The further systematic investigation of soils for yeasts promises rich results and new forms described may furnish data for the construction of a new more natural system of yeasts.

DIAGNOSIS.

Torulopsis pseudaria nov. spec.

In musto maltato cellulae globosae, 5–8 μ in diametro, singulae aut in "torulopsis"-cataenae. Post unum mensem, 20°C., sedimentum, anulus et insulae formantur.

In agaro maltato cellulae globosae sicut in musto maltato et cellulae apiculatae (10–12 μ) \times (4–6 μ) quoque. Cultura (post unum mensem, 20°C.) albida, parum nitens, prope plana.

Pseudomycelium nullum. Sporulatio nulla. Fermentatio nulla.

Glucosum, galactosum, saccharosum, maltosum, lactosum, melibiosum,

trehalosum, raffinose, dextrinum, arabinosum, xylosum, mannitum, sorbitum, dulcitur, acidum aceticum, citricum et gluconicum assimilantur, amyllum, inulinum, etanolum, glycerinum, acidum lacticum, succinicum, fumaricum, malicum, tartaricum non assimilantur.

Nitras kalicus assimilatur. "Amyllum" et pigmenta carotenoidica non synthetisantur. Esculinum infirmissime finditur.

Isolata ex solo vineae in monte Badacsony apud lacus Balaton, Hungaria.

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SACCHAROMYCES SMITTII NOV. SPEC.

A NEW YEAST ISOLATED FROM DUTCH, ITALIAN AND SWEDISH SOILS

by

AUGUSTO CAPRIOTTI

(Received April 21, 1958).

In November 1952 I isolated from Dutch (1954) and from Italian soils (1955a) some yeast strains that I identified as *Sacch. microellipsodes* (Ostenwalder) and one of which I sent in 1953 to the Yeast Division of C.B.S. at Delft (strain 31/A).

Successively other strains isolated by myself in 1955 from some Swedish soils (1957) were described as *Sacch. microellipsodes* var. *maltosa* and one was sent to Delft (strain 740). A more profound study led to the conclusion that these strains must be referred to as a new species¹⁾ for which I propose the name *Saccharomyces smittii* in memory of the friend and master JAN SMIT (late Director of the Laboratory for Microbiology at Wageningen (Holland)).

Origin of strains. 20 strains were investigated: 4 of them were found in the garden of the Laboratory for Microbiology at Wageningen (1954); 13 found in soils from vineyards, orchards and wheat fields of Central Italy, near Perugia (1955a); 3 in soils grown with potatoes in Ultuna, Uppland, Sweden (1957).

General methods.

For the determination of characters, the method of LODDER and KREGER-VAN RIJ (1952) was followed, except as to the methods concerning the study of the assimilation of sugars. For this determination I considered the use of washed agar more suitable (CAPRIOTTI, 1955b).

¹⁾ I am indebted to Mrs N. J. W. KREGER-VAN RIJ (Delft) for confirming this conclusion.

Description.

Growth in malt extract: after 3 days at 25°C. the cells are round to oval $(3.5-5.5) \times (4.5-7) \mu$, single or in pairs, budding (Fig. 1). Fermentation and sediment.

After one month at 17°C. there is a sediment, the liquid is transparent and a ring is more or less completely formed.

Growth in grape-must: after 3 days at 25°C., cells are round to oval, $(3.5-5.5) \times (4.5-7.3) \mu$, single or in pairs, budding. After one month at 17°C., sediment, clear liquid and ring more or less completely formed.

Growth on malt-agar: after 3 days at 25°C., cells are round and prevailing oval, $(3-6) \times (4-7.5) \mu$, single or in pairs, budding. Streak culture whitish.

After one month at 17°C., the streak culture is yellowish to grey-ochraceous, rather abundant, flat, glistening, waxen, margin smooth.

Growth on carrot-agar: after 3 days at 25°C., cells are round or elliptical, $(3.5-7.5) \times (3.6-8) \mu$, single or in pairs, budding. After one month at 17°C., the streak culture is whitish, glistening, scanty, flat and smooth.

Growth on potato-agar: after 3 days at 25°C., cells round which can attain $10-12 \mu$ in diameter, single or in pairs.

After one month at 17°C. the streak culture is white, greyish, waxen, glistening, rather abundant, margin smooth, surface smooth or slightly wrinkled.

Growth on bean-agar: after 3 days at 25°C., cells are round to elliptical, $(3-7) \times (3.5-8) \mu$, single or in pairs, budding.

After one month at 17°C. the streak culture is white-greyish, waxen, glistening, rather abundant, margin smooth, surface smooth or slightly wrinkled.

Growth on beef extract-agar: no growth.

Slide cultures: no pseudomycelium.

Growth on must-gelatine: single colonies are raised, whitish, no liquefaction.

Stab culture: after 45 days at 18°C., rather tardy liquefaction in the shape of an irregular funnel; the liquefied part is turbid and yellow-ochraceous in colour.

Growth in milk: no growth.

Sporulation: parthogenetical formation of asci which gene-

rally contain 4 spores of (2—2.5) μ in diameter. The walls of the spores are smooth (Fig. 2).

Fermentation:	glucose	+	lactose	—	
	galactose	+	raffinose	+	(complete)
	maltose	—	inuline	—	
	saccharose	+	dextrine	—	
Sugar assimilation:	glucose	+	saccharose	+	
	galactose	+	lactose	—	
	maltose	+	raffinose	+	

Assimilation of N compounds:				
	potassium nitrate	—		
	ammonium sulphate	+		
	asparagine	+		

Ethanol as sole source of carbon: fairly good growth with turbid liquid.

Splitting of arbutin: positive in some strains, but negative in others.

Fermentative power: in normal grape-must (pH 3.5) alcohol volume 3.12 to 5.14%.

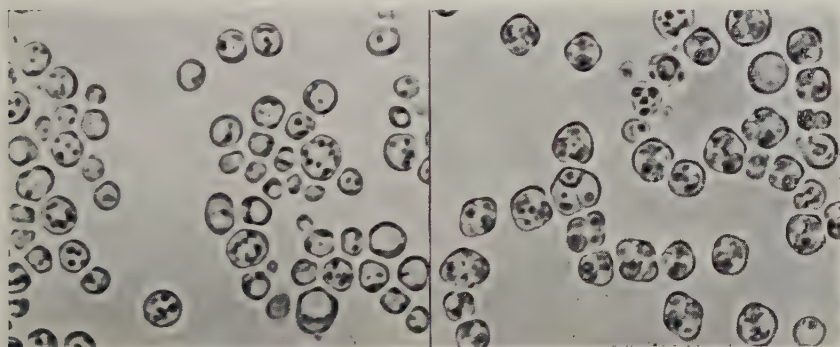


Fig. 1 and 2. *Saccharomyces smittii*.

DISCUSSION.

Considering that this micro-organism presents round and oval cells, parthogenetical asci (under normal microscopical observations) containing spores with smooth walls, ferments the sugars and does not assimilate potassium nitrate, it has to be referred to the genus *Saccharomyces*. The species up till now described belonging to this genus that, like *Saccharomyces smittii*, are capable of assimilating maltose but not of fermenting it, are the following: *Saccharomyces*

lactis Dombrowski, *Saccharomyces fructuum* Lodder et van Rij and *Saccharomyces veronae* Lodder et van Rij.

The characters distinguishing these species, together with those of *Saccharomyces microellipsodes* Osterwalder and of *Saccharomyces smittii* nov. spec. are indicated in table 1.

TABLE 1.

	<i>Saccharomyces lactis</i> Dombrowski	<i>Saccharomyces fructuum</i> Lodder et van Rij	<i>Saccharomyces veronae</i> Lodder et van Rij	<i>Saccharomyces microellipsodes</i> Osterwalder	<i>Saccharomyces smittii</i> nov. spec.
Dimension of the cells in malt-extract (μ)	(2.5-5) \times (3.5-9.5)	(3.5-7) \times (6-10)	(3-6) \times (3.5-6)	(3-5) \times (4-6.5)	(3-6) \times (4-7.5)
Fermentation:					
glucose	+	+	+	+	+
galactose	+	+	—	+	+
				(very weak)	
maltose	—	—	+	—	—
			(weak) or —		
saccharose	+	+	+	+	+
lactose	+	—	—	—	—
raffinose	+ 1/3	+ 1/3	+ 1/3	+	+
Assimilation:					
glucose	+	+	+	+	+
galactose	+	+	+	+	+
maltose	+	+	+	—	+
saccharose	+	+	+	+	+
lactose	+	—	—	—	—
raffinose	+	+	+	+	+
Potassium nitrate:	—	—	—	—	—
Ethanol as sole carbon source:	+	very weak or —	—	—	—
Arbutin:	+	—	—	—	+ or —
Fermentative power % in volume:		11.06-13.8	6.8-8.9		3.12-5.14

It is therefore obvious that, besides the shape and dimensions of the cells, *Saccharomyces smittii* nov. spec. differs:

from *Saccharomyces lactis* in that it does not ferment and assimilate lactose; ferments raffinose completely; does not show any growth in ethanol as sole source of carbon.

from *Saccharomyces fructuum* in that it ferments raffinose com-

pletely; does not show any growth in ethanol as sole source of carbon; often splits arbutin.

from *Saccharomyces veronae* in that it does not ferment maltose; ferments raffinose completely, splits arbutin (often).

Moreover the table shows the principal characters of *Saccharomyces microellipsodes* Osterwalder into which species *Saccharomyces smittii* nov. spec. was previously included as a variety with the name of *Saccharomyces microellipsodes* var. *maltosa*.

Saccharomyces smittii nov. spec. differs from *Saccharomyces microellipsodes* Osterwalder in that its cells are larger and in contrast with the above mentioned species assimilates maltose and often splits arbutin. Two strains of *Saccharomyces smittii* nov. spec. were sent to C.B.S. at Delft, whilst 11 strains were added to the yeast collection of the Institute of Agricultural and Technical Microbiology of the University of Perugia (Italy).

Saccharomyces smittii nov. spec.

In malto: cellulae rotundae ovoideae $(3.5-5.5) \times (4.5-7) \mu$, singulae vel binae geminatae. Mense elapso formatur sedimentum et corona plus minusve perfecta.

In agaro maltato: cellulae globosae-ovoidae, plerumque $(3-6) \times (4-7.5) \mu$, singulae vel binae geminatae. Post mensem pellicula alba-sufflava, vel flavescens, paene lutea, copiosa, aequae, levigata, cerea, levibus marginibus.

Pseudomycelium non formatur. In musteo concreto jure fit fluens admodum sero.

Asci partenoparientes 1—4 prediti sporis globosis, parietibus glabris. Plures sunt asci 4 sporas habentes.

Fermentatur glucosum, galactosum, saccharosum et raffinsum (totaliter). Assimilatur glucosum, galactosum, maltosum, saccharosum et raffinsum. Non assimilatur nitras kalicus. Praesente alcohole aethylico veluti unice carbonii fonte minime augetur. Varie arbutinum finditur. Alcohol aethylicum editum: magnitudine 3.12—5.14%.

Isolata est e terra.

S u m m a r y.

A new species of *Saccharomyces* is described, which was isolated from soils. The name *Saccharomyces smittii* is proposed in memory

of the late Prof. JAN SMIT (Director of the Laboratory for Microbiology, Wageningen, Holland).

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A NEW SPECIES OF *BELTRANIA* FROM SOIL

by

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(Received March 18, 1958).

In the course of an investigation of the fungus population of mangrove soils from the island of Inhaca (near Lourenço Marques, on the East Coast of Africa) a species of *Beltrania* was isolated. When an attempt was made to identify the culture some features were noticed, in this species, that do not occur, or are different from those in the five species of this genus described up to now (HUGHES, 1951; SUBRAMANIAN, 1952). Therefore the isolate will be described here as a new species.

Beltrania multispora nov. spec.

Coloniae in agar avenae albae pannis nigri pseudoparenchymatis formantibus ordines tenues in summo agar.

Setae erectae, sensim septatae, atrobrunneae, mediocriter crasse tunicatae, 150—250 μ longae, 3—4 μ latae in basi, apice acutae, plerumque emergentes ex basi conidiophori brevioris.

Conidiophora simplicia vel interdum ramosi in basi, in longitudinem inter se differentia, usque ad 250 μ , 3—4 μ lata, pallide-brunnea, apice interdum pallidiore maxime inaequalia quod proliferare solent, valde denticulata.

Cellulae separantes hyalinae, ovatae, utrimque denticulatae, 9—13 \times 4—5 μ .

Conidia inaequaliter biconica, continua, atrobrunnea, pallidebrunnea vel subhyalina zona super latissimam partem praedita, (17) 24—26 μ , \times 8 (9) μ , rotunda vel parvo 1-denticulata in basi, ferentes longam conicam 1 cellulam hyalinae setae in apice, 9 (6—10) μ longa et 1 μ lata in basi. Conidia recte affixa apici conidiophori et producta duo vel tria in cellulis separantibus.

Habitatio in solo harenoso prope paludem mangrovorum in Inhaca insula, Mozambique.

Beltrania multispora nov. spec.

Colonies on oatmeal agar white with patches of black pseudo-parenchyma forming thin layers on the agar surface.

Setae straight, erect, distantly and inconspicuously septate, dark

brown, fairly thick walled, 150 to 250 μ long, 3 to 4 μ wide at the base, tapering to an acute apex, usually arising from the base of a shorter conidiophore.

Conidiophores simple or sometimes branched at the base, rather variable in length, up to 250 μ , 3 to 4 μ wide, light brown, apex sometimes lighter, very irregular due to a definite tendency to proliferate and strongly denticulate.

Separating cells hyaline, oval, denticulate at both ends; 9 to 13 μ by 4 to 5 μ .

Conidia unequally biconic, unicellular, dark brown with a pale brown or subhyaline band just above the widest part, (17) 24–26 μ by 8 (9) μ , rounded or slightly 1-denticulated at the base, bearing a long-conical, 1-celled, hyaline seta at the apex 9 (6–10) μ long and 1 μ wide at the base. Conidia are both directly attached to the apex of the conidiophore and borne with 2 or 3 on the separating cells.

Habitat: in sandy soil near mangrove swamp, Inhaca island, Mozambique.

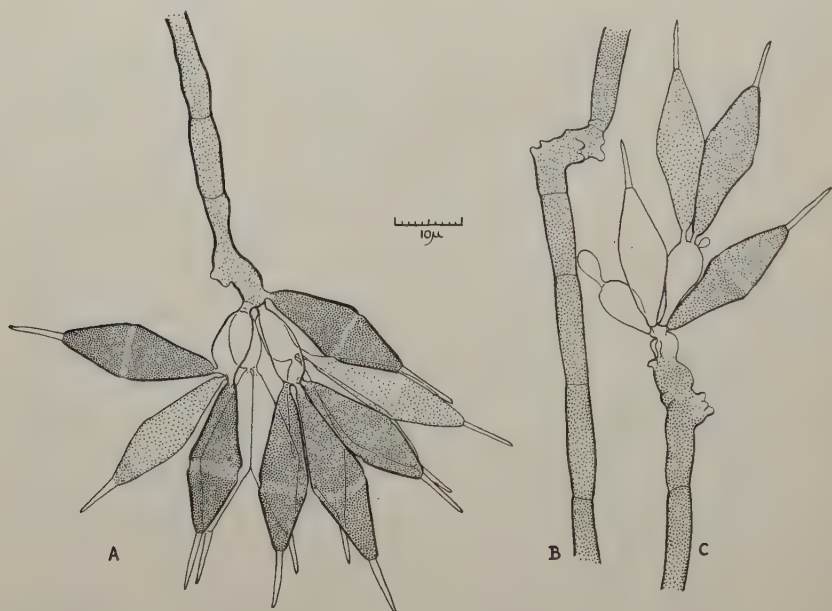


Fig. 1. *Beltrania multispora*. A: tip of conidiophore with conidia; B: fragment of nodose conidiophore; C: tip of conidiophore showing different stages in development of conidia.

Due to the production of several conidia per separating cell and the proliferation of the conidiophore the latter bears a very compact cluster of conidia when mature. The conidia on the separating cells develop one after the other, as is visible in fig. 1, C. Sometimes the proliferation of the conidiophore is so pronounced that it becomes nodose (see fig. 1, B).

It was noticed that in a dry condition the conidia are more or less flat and the hyaline part of the cell wall – which is noticeably thinner – is more or less sucked inwards. The conidium then looks 2-celled and constricted at its widest part (see fig. 2, A). This might explain PENZIG's observation of 2-celled conidia in his *B. rhombica* (see HUGHES, 1952).

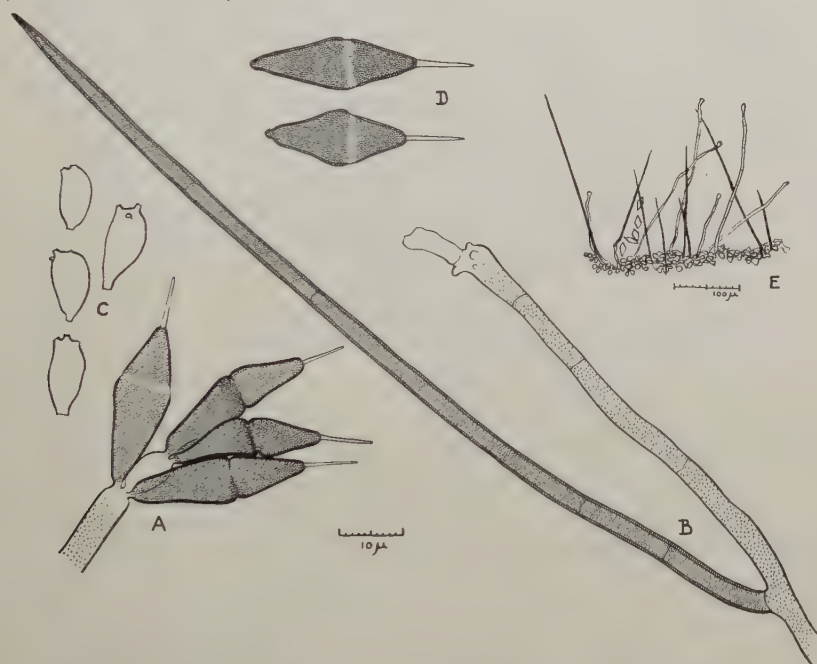


Fig. 2. A: tip of conidiophore in dry condition; B: conidiophore with setae; C: separating cells; D: conidia; E: conidiophores and setae as arising from pseudoparenchyma.

If we compare the above description with those of the other species of *Beltrania* it will be noticed that the production of more than one conidium per separating cell is the main distinctive feature of this new species. The conidia are closely similar in size to those of *B. rhombica* except for their somewhat longer setae. The prolife-

ration of the conidiophore is not mentioned but well illustrated in *Beltrania indica* (SUBRAMANIAN, 1952) and seems to occur to a certain extent in *B. africana* (HUGHES, 1951) as indicated by the illustration.

Cultures of the organism will be sent to the C.M.I., Kew, and the C.B.S. in Baarn.

A c k n o w l e d g e m e n t s.

The author is indebted to Professor S. DAVIS, Department of Classics, for the latin translation of the diagnosis and to Miss F. D. HANCOCK, M. Sc. for correcting the manuscript.

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ON THE CELL WALL COMPOSITION OF THE APICULATE YEASTS

by

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(Received May 12, 1958).

Interest in the composition of yeast cell walls has been exhibited by various investigators since SALKOWSKI (1894) first extracted the so-called yeast gum from bakers yeast. He found that upon hydrolysis yeast gum yielded glucose and mannose. Most of the work has been done with bakers yeast although there is a considerable amount of data published on other species of yeast.

The polysaccharides generally found in the cell walls are yeast mannan, yeast glucan and chitin. In addition lipid materials and proteins are present. Yeast mannan and yeast glucan usually make up the greater part of the cell wall material.

GARZULY-JANKE (1940) examined 139 species of fungi and yeasts for the presence of mannan. She used the criterion of formation of a copper-mannan precipitate as a positive indication that mannan was present. This was accomplished by the addition of Fehling solution to sulfuric acid extracts of the fungi examined. In the 12 genera of yeast included in her study she found mannan to be present in most species. Mannan appeared to be absent in cell wall preparations of *Nadsonia fulvescens*, *Rhodotorula* spp., and in *Schizosaccharomyces octosporus*, as well as in all hyphal fungi examined.

SCHMIDT (1936) was the first to claim that certain yeasts had chitin. ROELOFSEN and HOETTE (1951) investigated 32 strains of yeasts, representing 12 genera and 29 species, for the occurrence of chitin in their cell walls. They found that chitin was present in all yeasts examined with the exception of *Schizosaccharomyces octosporus*. The amounts were very small, varied with different species and seemed to increase in older cultures. They used a modified chitosan sulfate crystallization test for the detection of chitin.

Mechanical disruption of cells was facilitated when MICKLE (1948)

developed a tissue disintegrator employing Ballotini beads. This permitted the use of isolated cell walls for cell wall studies, rather than the chemical hydrolysis or extraction of whole cells as was used by earlier investigators. Material prepared by mechanical disruption is chemically intact and can be essentially freed of cell contents.

HOUWINK and KREGER (1953) prepared cell walls in this way and showed the presence of chitin in electron micrographs of partially digested cell walls of bakers yeast and of *Candida tropicalis*. In bakers yeast the chitin seemed to be located in the bud scars and possibly in a broad zone around the scars. In *Candida tropicalis*, however, the photomicrographs suggested that the chitin occurs throughout the entire cell wall. *C. tropicalis* was shown to have a comparatively high proportion of chitin, while bakers yeast had a very small amount.

NORTHCOTE and HORNE (1952) and NORTHCOTE (1954) working with cell wall material from mechanically broken cells of *Saccharomyces cerevisiae* found the composition of the cell wall to be 29 per cent glucan, 31 per cent mannan, 13 per cent protein, 8.5 per cent lipid material and 3 per cent ash. They believed the wall to be composed of 2 or more membranes, the outer one containing glucan and the inner one mannan and protein.

ROELOFSEN (1953), working with similarly prepared cell wall material of bakers yeast, found that 68 per cent of the cell wall was carbohydrate and 6 per cent was protein. Glucose and mannose were present in approximately equal amounts. He found that extraction for 30 minutes with hot (100°C.) 2 per cent NaOH was sufficient to extract all of the mannan present. Thirty per cent alkali was commonly used by earlier investigators to extract whole cells. In addition, he reported that *ca.* 25 per cent of the glucan in the cell wall material was solubilized and extracted with mannan by hot alkali. HOUWINK and KREGER (1953, pg. 20) also found this to occur.

FALCONE and NICKERSON (1956) found a total carbohydrate content of 84.4 per cent and a protein content of 6.7 per cent in the cell walls of bakers yeast.

KREGER (1954) used X-ray diffraction patterns, after alkali and/or acid treatment of cell wall material, for the detection of glucan and chitin. He was able to substantiate many of the conclusions of previous investigators regarding the presence or absence of these polysaccharides in various yeasts and other fungi.

Our present knowledge of the composition of cell walls may be summarized as follows: Yeast mannan is present in all of the budding yeasts with the exception of species of *Rhodotorula*, *Sporobolomyces* and *Nadsonia*. Yeast mannan is also absent in *Schizosaccharomyces*, in the filamentous yeasts (*Endomycopsis* has a small amount) and in those representatives of the *Phycomycetes* and *Euascomycetes* studied. Yeast glucan is present in all of the budding yeasts except members of the genera *Rhodotorula* and *Sporobolomyces*. It is also present in the filamentous yeasts. Chitin is present in all of the yeasts studied with the exception of species of *Schizosaccharomyces*. The amount of chitin present in most of the yeasts is very small. The mannan-deficient species have much larger quantities of this polysaccharide.

FREY (1950) has suggested that data on cell wall composition in fungi may have taxonomic significance. CUMMINS and HARRIS (1956, 1958) have shown the value of such data in the classification of the *Actinomycetales* and helped to clarify the systematic position of this order.

Among the genera of the tribe *Nadsonieae* (LODDER and KREGER-VAN RIJ, 1952) only *Nadsonia* has been examined to any extent with regard to its cell wall composition. As the cell walls of members of this genus lack mannan, a constituent found in most budding yeasts, it was felt desirable to determine the cell wall composition of *Hanseniaspora* and of *Saccharomycodes*, which comprise the other two genera in this tribe. It was felt that such information, in conjunction with morphological and physiological properties, might well contribute to a clarification of the relationship between these three genera and their taxonomic position.

MATERIAL AND GENERAL METHODS.

The cultures of yeasts used in this study were *Nadsonia elongata*, *Saccharomycodes ludwigii*, *Hanseniaspora valbyensis*, *H. uvarum* (Syn. *Kloeckeraspora uvarum*), *Kloeckera apiculata* and *Saccharomyces cerevisiae*. The cultures were maintained on 5° Balling malt agar. Cells used for the preparation of cell walls were grown on the same medium.

Total carbohydrates were determined by a modification of the anthrone method described by TREVELYAN and HARRISON (1951) and TREVELYAN *et al.* (1952). The modification consisted of heating

the anthrone-carbohydrate mixture in a boiling water bath for 2.5 minutes and cooling under running tap water for 5 minutes prior to reading the optical density. Measurements were made in a Klett-Summerson colorimeter using a red filter (No. 62). Excellent agreement was obtained by this procedure between duplicates within a series and also between standards of known carbohydrate concentrations of the various runs. Exact timing and duplication of conditions were found to be extremely critical. Glucose and mannose were used as standards.

Since the curves relating known concentrations of glucose and mannose to Klett units have different slopes it was necessary to interpolate between the curves to determine the carbohydrate content of mixtures of these sugars. The approximate ratio of glucose and mannose in a particular mixture was estimated by chromatographic spot intensities compared to those of various known concentrations of these sugars.

Total nitrogen was determined after digestion of the sample by direct nesslerization following the method described by JOHNSON (1941). Optical density was measured in a Klett-Summerson colorimeter using a blue filter (No. 42). Ammonium chloride was used to prepare nitrogen standards.

Dry weight determinations of the cell walls were made in small aluminum moisture dishes. Drying was done in a vacuum oven at 70°C. until a constant weight was obtained (*ca.* 8 to 12 hours).

Hydrolysis of cell wall material was accomplished in sealed glass tubes with 2 N HCl by autoclaving for 30 minutes at 121°C. The hydrolysate was evaporated to dryness under vacuum over KOH pellets. Water was added to the residue so that the approximate concentrations of sugars were 500 to 1000 gamma per ml. Chromatography was done with 0.05 ml portions of this material.

The separation of sugars was accomplished on unidirectional descending chromatograms. Whatman paper No. 1 was used. The best separation of sugars was obtained with isopropanol: ethanol (95 per cent): water (7:2:1) as the solvent. Detection of the sugars was done by spraying with a silver nitrate saturated acetone solution followed by a spray of alcoholic sodium hydroxide (TREVELYAN and HARRISON, 1951). Meta-phenylenediamine in alcohol was used as a spray to detect reducing sugars. Amino acids were separated on a bi-directional descending chromatogram. Phenol: water (2:1) and butanol: acetic acid: water (4:1:5) were the solvents used. Ninhydrin

(0.1 per cent in acetone) was used to detect the amino acids and glucosamine.

PREPARATION OF CELL WALLS.

A. Rupturing of cells by sonic oscillation: Sonic oscillation of cell suspensions has been used for the purpose of obtaining cell-free enzyme preparations. This method of cell disruption was applied in this study to obtain cell wall material. Discussions on the mechanics of rupturing whole cells by ultra-high frequency sound waves may be found in a review by HUGO (1954) and in an article on sonic disruption of *Azotobacter* by MARR and COTA-ROBLES (1957).

A study was made to determine the relation between the time of sonication, changes in the turbidity of the suspension, observable breakage of cells and the release of nucleic acids into the medium by cells of *Hanseniaspora uvarum*. The cells used in this experiment were grown for 3 days on malt agar, harvested, and washed 3 times with distilled water. A light suspension was used in this study so that changes in turbidity could be read on the linear portion of a curve relating Klett units to concentrations of cells, using a Klett-Summerson colorimeter with a blue filter (No. 42). Counts of the intact cells were made in a Spencer phase chamber. Phase microscopy was used to determine whether or not a cell was broken because of the greater degree of contrast obtainable between intact and empty cells. Cells were judged to be intact if they gave a high contrast. The release of nucleic acids was determined by centrifuging aliquots of the sonicate at 2000 xG for 10 minutes and measuring the optical density values at 260 m μ (E_{260}) in a Beckman spectrophotometer. A Raytheon sonic oscillator (magnetostriction type), operating at 10 kilocycles, 250 watts, using maximum plate current (1.30—1.35 amperes) was used to disrupt the cells. The temperature of the transducer was *ca.* 2°C. and the atmosphere over the cell suspension was flushed for 3 minutes with hydrogen prior to sonication.

Data obtained on the rate of cell breakage by sonic oscillation are shown in Figure 1. Data obtained in the same manner with cells of *Saccharomyces cerevisiae* are shown for comparison in Figure 2.

Fifty per cent of the *Hanseniaspora* cells appeared to be broken in *ca.* 6 minutes when examined by phase microscopy. An oscillation

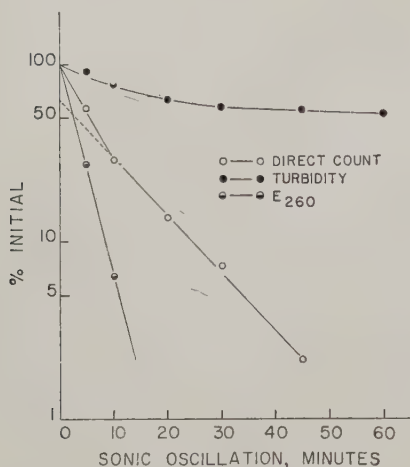


Fig. 1. Rate of breakage in cells of *Hanseniaspora uvarum* by sonic oscillation.

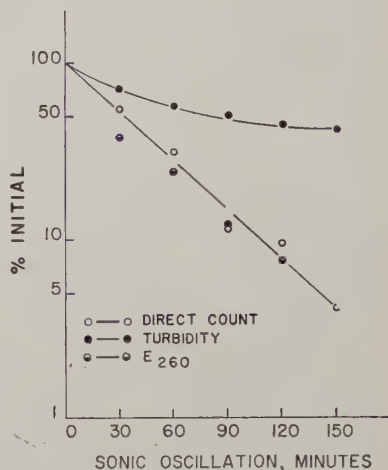


Fig. 2. Rate of breakage in cells of *Saccharomyces cerevisiae* by sonic oscillation.

time of 32 minutes was required for the same amount of breakage of cells of *S. cerevisiae*.

Cell suspensions of *Hanseniaspora* are not homogeneous with respect to cell size and were found to contain about 32 per cent of small ovoid cells. The difficulty of recognizing breakage in these small cells may be responsible for the break in the count of intact cells which occurs near 70 per cent breakage. On the other hand, the release of nucleic acids from the cells, as indicated by an increase in E_{260} values in the supernatant liquid, is linear and occurs at a more rapid rate than is shown by the decrease of the count of intact cells. This indicates that the cell breakage and subsequent loss of the cell contents occurs at a faster rate than can be observed directly. The direct count of intact cells and the rate of nucleic acid release coincided when *S. cerevisiae* was used. The larger cell dimensions and the much greater uniformity of cell size in this species may be the reason for this agreement.

A 30-minute period of oscillation was chosen for the preparation of cell wall material. At this time, *ca.* 93 per cent of the cells are broken and the majority of the cell walls are not excessively fragmented. Batches of cells up to 10 grams wet weight were suspended in 50 ml of water and ruptured to obtain cell wall material. At this concentration the percentage of cell breakage is similar to that observed in the more dilute suspension.

B. Purification of the cell wall material: After sonic rupture of the cells, the cell wall material was purified by the following procedure: The sonicate was centrifuged at 4400 xG for 10 minutes. The sediment was resuspended in water and again centrifuged at 4400 xG and finally at 3100 xG for periods of 10 minutes. The sediment, consisting mainly of cell walls and whole cells, was then transferred into a 15 ml conical tube and suspended in 10 ml water. This suspension was centrifuged at 4000 r.p.m. for 10 minutes in a clinical table model centrifuge. The supernatant, containing some granules and a small amount of cell walls (very small fragments), was discarded. The sediment consisted of two layers. The upper layer was fluffy, white and consisted mainly of cell walls. The lower layer was buff and compact and contained mainly whole cells. The upper layer was removed by gently suspending it in a small amount of water and transferring it to another tube. This procedure was repeated until the separation of the two layers was as complete as possible and the supernatant liquid over the cell wall fraction was free of particles. This required *ca.* 50 centrifugations. The yield of cell wall material is about 5 per cent on the basis of the original wet weight of whole cells. This material contained 6.2 per cent dry matter; it is light and fluffy, white in color, free of cytoplasmic particles and virtually free of whole cells. A small amount of protoplasm might adhere to the interior of the larger cell fragments although microscopic examination did not disclose any observable inclusions. The cell wall material upon staining was Gram negative. Whole cells stain Gram positive.

Small batches of cell wall material of *H. valbyensis*, *Kloeckera apiculata*, *Saccharomycodes ludwigii* and of *Nadsonia elongata* were prepared in a similar way. The cell wall material was stored at 4°C. until analyzed.

ANALYSIS OF THE CELL WALLS.

Chromatographic analyses of hydrolyzed cell wall material showed that *Hanseniaspora valbyensis*, *H. uvarum*, *Kloeckera apiculata* and *Saccharomycodes ludwigii* gave similar chromatographic patterns. All contained glucose and mannose in approximately equal amounts and in addition very small amounts of glucosamine. Control experiments with hydrolyzed cell wall material of *Saccharomyces cerevisiae* gave a similar pattern as that obtained with the apiculate

yeasts. Chromatograms of hydrolyzed cell wall material of *Nadsonia elongata* showed glucose and glucosamine to be present, but mannose was absent. The glucosamine was present in much larger amounts than in the cell wall hydrolysates of the apiculate yeasts or of *S. cerevisiae*.

Since the chromatograms of the three apiculate yeasts and of *Saccharomyces* were the same, only cell wall material obtained from *H. uvarum* was fractionated and the various fractions analyzed.

Method of fractionation of cell wall carbohydrates.

The procedure used in this study is based on the methods of NORTHOTE and HORNE (1952), of ROELOFSEN (1953) and of HAWORTH *et al.* (1937). The method used is as follows:

I. Dry weight, total carbohydrate and nitrogen content.

Two portions of *ca.* 100 mg wet cell wall paste were taken for duplicate dry weight determinations. Approximately 500 mg of clean cell wall material (wet weight) was brought to a total volume of 10 ml with distilled water in a volumetric flask. For the determination of the total carbohydrate content and of the total nitrogen content, aliquots of 0.05 ml and 0.20 ml were used, respectively.

II. Extraction of the yeast mannan.

The remaining suspension of cell wall material (I) was quantitatively transferred to a 15 ml conical centrifuge tube and centrifuged at high speed. The clear supernatant was discarded and 3 ml of 3 per cent NaOH (w/v) were added to the cell wall sediment. This was placed in a boiling water bath for 15 minutes. A stirrer made of a thin glass rod was employed to keep the suspension constantly agitated. This preparation was centrifuged, the supernatant decanted and the extraction repeated with a fresh portion of NaOH. The residue was washed twice with 4 ml portions of water. The supernatant extracts and the washings were combined, the pH adjusted to 6.5 with 10 per cent acetic acid (1.7 ml used), and the volume made to 25 milliliters with water. Aliquots of 0.25 ml were taken for determination of the total carbohydrate content, and 1.00 ml for chromatographic analysis.

III. Precipitation of the crude yeast mannan.

Eighty eight milliliters of 95 per cent ethanol were added to 22 ml of the alkali extract (II). The precipitated mannan was centrifuged at high speed. The supernatant liquid was discarded and

the sediment dissolved in 2 ml water. Hot water was necessary to dissolve the sediment completely. The mannan was precipitated again by the addition of 8 ml ethanol (95 per cent) and recentrifuged. The resultant white sediment was then washed twice with 95 per cent ethanol and once each with absolute ethanol and ether and dried.

IV. Purification of the yeast mannan.

Three milligrams of the crude mannan (III) were dissolved in 4 ml warm water and 12 ml Fehling solution added. This resulted in a flocculent precipitate. After 30 minutes the precipitate was centrifuged. The sediment was compact and blue-gray in color. The precipitate was washed 3 times with 5 ml portions of water and then resuspended in 3.0 ml water. Six normal hydrochloric acid (0.05 ml used) was added until the precipitate dissolved completely. Nine milliliters of ethanol were added to this solution resulting in a milky white suspension. After centrifugation the white sediment was washed twice with 95 per cent ethanol. The sediment dissolved completely in 1 ml cold water. The mannan was reprecipitated with 3 ml ethanol (95 per cent), centrifuged and washed with 3 ml ethanol (95 per cent), absolute ethanol, and ether. The white mannan residue was then dried.

V. Purification of the yeast glucan.

The insoluble residue after alkali extraction (step II) was treated with 2 ml 0.5 N acetic acid for 15 minutes at 75°C. with constant agitation. After centrifugation, the sediment was washed twice with 3 ml portions of distilled water, 70 per cent and 95 per cent ethanol and once each with absolute ethanol and ether. The glucan residue was then dried.

VI. Measurement of cell wall chitin.

The amount of chitin present in the cell wall material was inferred from a determination of the glucosamine content. This was determined by the method of ELSON and MORGAN (1933) as modified by BERGER and REYNOLDS (1958).

VII. Chromatography of the cell wall fractions.

The unfractionated cell wall material, the cell wall fractions from step II (alkali extract), step III (crude mannan), step IV (purified mannan), and step V (purified glucan) were hydrolyzed with acid and chromatographed. All analyses were done in duplicate. Results were in close agreement.

Results of the fractionation procedure.

Whole cell wall material.

Chromatography. The sugars detected were glucose and mannose, in approximately equal amounts, and a weak but definite spot for glucosamine.

Only eight amino acids could be identified with certainty. These were as follows: Aspartic acid, glutamic acid, serine, glycine, threonine, alanine, lysine (weak), leucine and/or iso-leucine, and probably valine. Three additional spots were detected but not identified.

Analytical.

Total amount of cell walls used	30.3 mg (dry weight)
Total carbohydrates (assuming <i>ca.</i> 50 per cent mannose and 50 per cent glucose)	78.1 %
Total protein ($6.25 \times$ N content)	7.0 %
Chitin content	<i>ca.</i> 0.05%

Alkali extract.

Chromatography. The sugars detected consisted of a very large amount of mannose and a small amount of glucose. Using various known concentrations of mannose and glucose, it was estimated that this fraction contained 1 glucose unit to 3 units of mannose. No glucosamine was present. The amino acids present were similar to those in the whole cell wall material.

Analytical. Total carbohydrate determined by anthrone showed that 63.1 per cent of the carbohydrate content of the cell wall material was extracted.

Crude mannan precipitate.

Chromatography. The chromatograms were the same as those obtained with the hydrolysate of the alkali extract.

Analytical. Total amount of dry precipitate was 15.4 mg or 52.3 per cent of the original whole cell wall material. Of the carbohydrates present in the alkali extract, 92.6 per cent was precipitated by ethanol (assuming 3 mannose units to 1 glucose unit in composition). The total protein content was 10.6 per cent.

Purified mannan.

Chromatography. The purified mannan consisted nearly completely of mannose. Only a very faint spot of glucose was observed. Amino acids were virtually absent on the chromatograms.

Analytical. Total amount of dry precipitate was 10.5 mg or 34.9 per cent of the original cell wall material used. Total carbohydrate content of this fraction was 99.8 per cent assuming only mannose to be present. Only a trace of protein remained.

Purified glucan.

Chromatography. Only glucose was present. Amino acids were absent.

Analytical. The dried purified glucan weighed 5.9 mg. The amount of glucan solubilized by alkali should be added to this. This was considered to be the weight of crude mannan minus the combined weights of the protein present in the crude mannan and the weight of purified mannan = $15.4 - (1.6 + 10.5) = 3.3$ mg. Thus the total glucan amounted to 9.2 mg or 30.4 per cent of the original cell wall material used. Total carbohydrate content of the purified glucan was 102 per cent assuming only glucose to be present. No protein was found in this fraction.

The recovery of carbohydrates (mannan plus glucan) was 83.3 per cent of the total carbohydrates originally present in the cell wall material.

As a check on the methods used some analyses were made on similarly prepared cell walls of *Saccharomyces cerevisiae*. Analyses showed the total carbohydrate content to be 82.8 per cent and the protein content to be 6.5 per cent. The chitin content was *ca.* 0.04 per cent.

Cell walls of *Nadsonia elongata* contained *ca.* 5 per cent of chitin.

DISCUSSION.

Cell wall composition data are becoming more and more useful as a tool in microbial systematics. This is illustrated by the work of AVERY and BLANK (1954) and of CUMMINS and HARRIS (1956, 1958) who worked with species of the *Actinomycetales*, *Mycobacteriaceae* and certain other Gram positive bacteria.

Of the genera in the tribe *Nadsonieae* only *Nadsonia fulvescens*

has been studied to any extent. The only published information on the cell walls of *Saccharomyces ludwigii* states that chitin is present (NABEL, 1935). The literature regarding the cell wall composition of *Hanseniaspora* or *Kloeckera* is limited to the findings of GARZULY-JANKE (1940) that cells of *K. apiculata* contain mannan.

The authors have shown that *N. elongata* has the same qualitative cell wall composition as the previously studied *N. fulvescens*. It is lacking in mannan and contains a relatively large amount of chitin. It was also demonstrated that the cell wall composition of *Saccharomyces ludwigii*, *Hanseniaspora uvarum*, *H. valbyensis* and *Kloeckera apiculata* is very similar. Approximately equal amounts of glucose and mannose account for nearly all of the carbohydrates found in the cell wall. Glucosamine was only detected in trace amounts. These sugars are indicative of the presence of the polysaccharides glucan, mannan and chitin, respectively. No other sugars were detected on chromatograms of the cell wall hydrolysates. These qualitative results are very similar to those reported for *Saccharomyces cerevisiae* in the literature and confirmed in this study. Quantitative analyses of the cell walls of *H. uvarum* showed that their composition is in close agreement with the values reported in the literature for cell wall material of *S. cerevisiae* (NORTHCOTE and HORNE, 1952; ROELOFSEN, 1953; FALCONE and NICKERSON, 1956).

On the basis of the composition of the cell wall the tribe *Nadsonieae* appears to be composed of two related genera and one unrelated genus. *Nadsonia*, because of the absence of mannan and of the relatively large amount of chitin in its cell wall, appears to be more closely related to the genus *Endomyces*. Species of *Saccharomyces* and *Hanseniaspora*, on the other hand, seem to be related to *Saccharomyces cerevisiae* as the cell wall compositions of these yeasts are very similar.

When STELLING-DEKKER (1931) separated *Nadsonia*, *Saccharomyces* and *Hanseniaspora* from the *Saccharomycetaceae* and placed them in the tribe *Nadsonieae*, she based this separation on the manner in which the vegetative cells reproduced, *i.e.* bipolar budding on a more or less broad base. *Saccharomyces* and *Hanseniaspora* bud on a relatively narrow base whereas *Nadsonia* produces buds on a very broad base approaching fission. The thallus of *Endomyces* reproduces exclusively by fission of the mycelium.

In addition, *Nadsonia* forms its ascospores in a manner very

different from *Hanseniaspora* and *Saccharomyces* (cf. PHAFF and MRAK, 1948). The last two genera sporulate in a way that is common to numerous species of the genera comprising the tribe *Saccharomyceteae*.

The above considerations suggest that the apiculate yeasts belonging to *Hanseniaspora* and *Saccharomyces* are closely related and may be derived from certain species in the *Saccharomyceteae*. *Nadsonia*, on the other hand, appears to have a different origin and has certain features in common with members of the sub-family *Endomycetoideae*.

Although our present knowledge of cell wall composition of the yeasts is still quite fragmentary, the authors believe that additional studies with other species will provide supplementary information useful in supporting phylogenetic postulations. Such data may also help to clarify the taxonomic position of certain groups or species of yeasts.

S u m m a r y.

Sonic oscillation was used for the purpose of obtaining clean, chemically intact cell walls. The rate of disruption was determined for cells of *Hanseniaspora uvarum* and *Saccharomyces cerevisiae*.

The carbohydrate fractions of cell walls of *Hanseniaspora uvarum*, *H. valbyensis*, *Kloeckera apiculata*, *Saccharomyces ludwigii* and *Saccharomyces cerevisiae* were shown to be similar. Chromatography of cell wall hydrolysates of all these species demonstrated that glucose and mannose were the only sugars present (in about equal amounts) besides traces of glucosamine.

The cell walls of *H. uvarum* contained 78.1 per cent carbohydrates, 7 per cent protein and approximately 0.05 per cent of chitin. Fractionation of the polysaccharides lead to a recovery of 83.3 per cent of the carbohydrates present (30.4 per cent glucan and 34.9 per cent mannan).

Saccharomyces cerevisiae cell walls were found to have a carbohydrate content of 82.8 per cent, 6.5 per cent protein and a trace of chitin (0.04 per cent).

Nadsonia elongata contained a relatively large amount of chitin (ca. 5 per cent) and lacked mannan in its cell walls.

It was concluded that *Hanseniaspora* and *Saccharomyces* are closely related to the *Saccharomyceteae* but they have little in common with species of *Nadsonia*.

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THE WINE YEASTS OF THE CAPE

PART I. — A TAXONOMICAL SURVEY OF THE YEASTS CAUSING TURBIDITY IN SOUTH AFRICAN TABLE WINES

by

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INTRODUCTION.

Wine being a product of fermentation, the presence of viable cells of wine yeasts in young unfortified wines is by no means uncommon. While such cells generally die off during maturation, it is known that yeast cells may remain viable in wines over extremely long periods. GAYON and DUBOURG (1912) reported the presence of viable fermentative yeasts in seventy-two-year-old wines from the celebrated Château Lafitte. CASTOR (1952) similarly reported such yeasts from bottled wines, no less than eighty-eight years old from the same cellars. Quoting CASTOR (1952) "Few of these red Bordeaux were considered spoiled, although many were probably *passés* solely by reason of extreme old age". Nevertheless, it is a fairly common and recurrent problem in the manufacture of wines of all classes, and particularly in the case of white table wines, that secondary yeast development occurs to the detriment of quality. This development may either result in the formation of unacceptable hazes, or sediments, or both, or may give rise to the formation of undesirable flavours, and even result in a vigorous secondary fermentation.

The widespread occurrence and gravity of this type of wine spoilage has stimulated its study in most wine-producing areas. As early as 1897, the French investigator DUBOURG reported on the yeast turbidity of Sauternes wines and identified the causative organism as a *Zygosaccharomyces* species. Subsequently the pro-

blem was studied in France by, amongst others, GAYON and DUBOURG (1912), PEYNAUD and DOMERCQ (1955), PEYNAUD (1956) and DOMERCQ (1956). KROEMER and KRUMBHOLZ (1931) and KARAMBOLOFF and KRUMBHOLZ (1931) reported on the yeast turbidities in Portuguese and German wines. Other reports on yeast turbidities in German wines were made by ZIMMERMANN (1938, 1939), VOGT (1945) and SCHANDERL and DRACZYNSKI (1952). NECHAEV (1939) reported on this problem in Russian wines. In America the problem has also been studied extensively by CRUESS (1918), BAKER (1936), BAKER and CRUESS (1937), MRAK and MCCLUNG (1940), NICKERSON (1943), PHAFF and DOUGLAS (1944), CASTOR (1948, 1951, 1952), PARSONS (1949), and SCHEFFER and MRAK (1951).

However, due to the unsatisfactory state of yeast taxonomy at the time, many of the older investigations were seriously handicapped in that a systematic taxonomical treatment of the causative organisms was not possible. This has often frustrated subsequent efforts to correlate the role of such inadequately described organisms with any specific biochemical feature or with any particular type of spoilage. The challenge of a more exacting taxonomical approach to the problem has, however, been met in America by SCHEFFER and MRAK (1951) and in France particularly by PEYNAUD and DOMERCQ (1955, 1956).

Although several methods for the control of this type of spoilage have been advocated, only two have found any great industrial application, *viz.*, high concentrations of sulphur dioxide and filtration through either clarifying or sterilising filters. Both these methods have only limited scope. Experience has shown that some of the causative organisms resist the action of sulphur dioxide even at concentrations which impart an objectionable odour and flavour to the wines. While filtration is practicable when handling smaller quantities of wine which are not very heavily infected with yeast, there are limits to its feasibility when handling large quantities. The process becomes especially costly when this failure appears in bulk export wine shipments to destinations not equipped to handle large-scale filtration.

In South Africa, as in other wine producing lands, the wine industry has long been acquainted with the sporadic development of yeast hazes. This problem, however, has become more prominent, particularly during the last decade. Moreover, whereas previously

it was mainly dry white wines that were affected, dry red wines are now showing this defect to an increasing extent.

As a first step in the systematic investigation of the problem in South Africa, an extensive taxonomic survey of the causative organisms has been undertaken.

MATERIALS AND METHODS.

Sixty samples of wines showing yeast turbidity were investigated. These included chiefly dry white wines, although semi-sweet white, medium sparkling wines and pale dry sherry wines were also studied. One sample of a dry red wine and nine samples of newly fermented wines in tanks were also examined.

The wines were allowed to stand until sedimentation had taken place. In the case of bottled wines, the mouth of the bottle was first washed and then swabbed with spirit. The clear supernatant was decanted to leave a residue of *ca* 100 ml in which the sediment was taken up. This was transferred aseptically to a sterile, cotton-plugged centrifuge bottle and the sediment collected at 2,000 — 3,000 r.p.m. for 15 minutes. The clear supernatant was discarded, leaving about 1—2 ml of fluid and sediment. After resuspension, the sediment was examined microscopically and isolations of yeasts made from it.

As the isolation of organisms from clouded wines sometimes affords difficulty, isolations from each wine were attempted on a solid as well as in a liquid medium.

Isolation on solid medium.

The thick suspension of the sediment was directly plated on:

M e d i u m I

Glucose	50 g
Malt extract	3 g
Bactopeptone	5 g
Bacto yeast extract	3 g
Calcium carbonate	5 g
Agar	20 g
Tap water	1,000 ml
No adjustment of the pH was made.	
Sterilisation: 15 lb, 15 min.	

The inoculated plates were incubated aerobically at 25° C. for at least two weeks. During the incubation, precautions had to be taken to prevent mould infections which arose either from air-

borne spores or from mycelium fragments or spores present in the wines. Two types of yeast colonies were found to develop on Medium I:

a) Colonies which developed fairly rapidly — 2 to about 4 days — with moderate or no acid production.

b) Colonies which developed very slowly — 6 to about 14 days — with intense acid production and the solution of the calcium carbonate from the medium.

Yeast colonies of type *a*) were brought into pure culture by repeated plating on:

M e d i u m II

Glucose	10 g
Malt extract	3 g
Bactopeptone	5 g
Bacto yeast extract	3 g
Agar	20 g
Tap water	1,000 ml
No adjustment of the pH was made.	
Sterilisation: 15 lb, 15 min.	

Yeasts of type *b*) were first transferred directly to slants of Medium II, which had been supplemented with 20 g calcium carbonate per litre. When sufficient growth had occurred after incubation at 25° C., pure cultures were prepared by repeated plating on the same medium. Stock cultures were also maintained on this medium.

I s o l a t i o n i n l i q u i d m e d i u m .

An aliquot of about 1 ml of the thick suspension collected by centrifugation of the wine was transferred directly to 100 ml Erlenmeyer flasks containing 30 ml aliquots of

M e d i u m III

Glucose	10 g
Malt extract	3 g
Bactopeptone	5 g
Bacto yeast extract	3 g
Tap water	1,000 ml

The pH was adjusted to 4.0 with 10% tartaric acid.

Sterilisation: 15 lb, 15 min.

Liquid enrichment cultures were incubated for 2 weeks at 25° C. Plate cultures were subsequently prepared from the enrichments by direct plating on Medium I. Pure cultures were prepared as described above.

Generally, no significant differences were observed between the isolations obtained by plate and liquid enrichments. The plate method has the advantage of being less time-consuming.

Pure cultures obtained were identified and classified according to the standard techniques of LODDER and KREGER-VAN RIJ (1952) and WICKERHAM (1951).

RESULTS.

The results are recorded in Tables 1—6. From these it will be seen that the most frequently occurring yeast species in the South African wines examined are: 1) *Brettanomyces* species — 50% incidence; 2) *Saccharomyces oviformis* — 20% incidence; 3) *Saccharomyces acidifaciens* — 18% incidence; 4) *Saccharomyces cerevisiae* — 15% incidence, and 5) *Pichia membranaefaciens* — 13% incidence.

DISCUSSION.

The incidence of the significant species will be discussed separately.

1) *Brettanomyces* species. — The strains brought to this genus have not been classified in individual species. A detailed biochemical and taxonomical treatment of these strains will be the subject of a separate communication. All strains brought to this genus are, however, characterised by their slow growth, the absence of ascospore formation, the production of pseudomycelium, the formation of more or less 'ogive' cells, the presence of a marked fermentative dissimilation and the marked production of acetic acid on sugar-containing media under aerobic conditions.

An infestation incidence of 50% by *Brettanomyces* species in the wines examined confirms the views expressed by NIEHAUS (1958) who first drew attention to the possible occurrence of these yeasts in South African wines. The concern expressed by this author seems well-founded, since, as far as figures are available, this is the highest incidence ever reported for these yeasts associated with turbidity of bottled wines. Moreover, these yeasts appear capable of infecting almost all unfortified wine types.

TABLE 1.
Summary of results for all wines.

	Red wine	White wine	Total	% wines infected
Number of wines examined	1	59	60	
Number of yeast strains isolated	1	84	85	
Number of yeast species isolated	1	15	15	
<i>Brettanomyces</i> species	1	29	30	50
<i>Saccharomyces oviformis</i> Osterwalder 1924	—	12	12	20
<i>Saccharomyces acidifaciens</i> (Nickerson) Lodder et Kreger- van Rij 1943	—	11	11	18
<i>Saccharomyces cerevisiae</i> Hansen 1883	—	9	9	15
<i>Pichia membranaefaciens</i> Hansen 1888	—	8	8	13
<i>Saccharomyces cerevisiae</i> var. <i>tetra-</i> <i>sporus</i> (Beij.) Dekker 1931	—	3	3	5
<i>Candida mycoderma</i> (Reess) Lod- der et Kreger-van Rij 1870	—	3	3	5
<i>Debaryomyces vini</i> Zimmermann 1938	—	2	2	<5
<i>Debaryomyces hansenii</i> (Zopf) Lod- der et Kreger-van Rij 1889	—	1	1	<5
<i>Saccharomyces italicus</i> Castelli 1938	—	1	1	<5
<i>Saccharomyces elegans</i> Lodder et Kreger-van Rij 1952	—	1	1	<5
<i>Torulopsis bacillaris</i> (Kroemer et Krumbholz) Lodder 1931	—	1	1	<5
<i>Candida mellinii</i> Diddens et Lod- der 1942	—	1	1	<5
<i>Cryptococcus laurentii</i> (Kufferath) Skinner 1920	—	1	1	<5
<i>Cryptococcus diffluens</i> (Zach) Lod- der et Kreger-van Rij 1934	—	1	1	<5

Although the occurrence of *Brettanomyces* species in wines appears to be a problem of more recent times, this may be the result of more exact taxonomic studies. While the occurrence of yeasts belonging to the genus *Brettanomyces* was reported in beer as early as 1904 by CLAUSSEN, it was only in 1940 that CUSTERS showed that the yeast *Mycotorula intermedia* isolated by KRUMBHOLZ and TAUSCHANOFF in 1933 from French grape must was in

TABLE 2.

Summary of results for dry white wines.

Number of wines examined 37, number of yeast strains isolated 57,
number of yeast species isolated 12.

Organism	Incidence	% wines infected	% reported by DOMERCQ ¹⁾
<i>Brettanomyces</i> species	18	48.7	0
<i>Saccharomyces acidifaciens</i>	10	27.0	34.6
<i>Saccharomyces oviformis</i>	9	24.3	51.9
<i>Pichia membranaefaciens</i>	6	16.2	Not reported
<i>Saccharomyces cerevisiae</i>	4	10.8	23.1
<i>Candida mycoderma</i>	3	8.1	<2
<i>Saccharomyces cerevisiae</i> var. <i>tetrasporus</i>	2	5.4	} Not reported
<i>Debaryomyces vini</i>	1	<5	
<i>Debaryomyces hansenii</i>	1	<5	
<i>Torulopsis bacillaris</i>	1	<5	
<i>Cryptococcus laurentii</i>	1	<5	
<i>Cryptococcus diffluens</i>	1	<5	

¹⁾ % Wines infected reported by DOMERCQ for 52 samples of white wines from the Gironde area (DOMERCQ 1956).

TABLE 3.

Results for semi-sweet white wines.
(5 samples).

Organism	Incidence
<i>Brettanomyces</i> species	2
<i>Saccharomyces acidifaciens</i>	1
<i>Pichia membranaefaciens</i>	1
<i>Cryptococcus laurentii</i>	1

TABLE 4.

Results for pale dry sherries.
(3 samples).

Organism	Incidence
<i>Brettanomyces</i> species	1
<i>Saccharomyces cerevisiae</i>	1
<i>Saccharomyces oviformis</i>	1
<i>Saccharomyces cerevisiae</i> var. <i>tetrasporus</i>	1

TABLE 5.
Results for medium-sweet sparkling wines.
(5 samples).

Organism	Incidence
<i>Brettanomyces</i> species	2
<i>Saccharomyces cerevisiae</i>	1
<i>Candida mellinii</i>	1
<i>Debaryomyces vini</i>	1
<i>Saccharomyces elegans</i>	1

TABLE 6.
Results for newly-fermented wines in tanks.
(9 samples).

Organism	Incidence
<i>Brettanomyces</i> species	6
<i>Saccharomyces cerevisiae</i>	3
<i>Saccharomyces oviformis</i>	3
<i>Saccharomyces italicus</i>	1

reality identical with *Brettanomyces bruxellensis* (Kufferath et van Laer). Similarly, SCHANDERL and DRACZYNSKI (1952) and SCHANDERL (1950) pointed out that the organism isolated by OSTERWALDER (1912) from apple wine and described by him as *Monilia vini*, was probably a *Brettanomyces* species. On the other hand, yeasts isolated from vinous products and classified as *Brettanomyces* species were deleted from this genus on re-examination [LODDER and KREGER-VAN RIJ (1952)].

The first uncontested account of the occurrence of these yeasts in bottled wines was by SCHANDERL (1950) and SCHANDERL and DRACZYNSKI (1952), who reported them as causing not only a troublesome turbidity, but also a marked increase of the volatile acid content of the German Sekt wines. AGOSTINI (1950) also described the isolation of a yeast from wines which had shown acetic acid spoilage in the absence of bacterial infection. Although he classified it as a *Brettanomyces*, the issue is somewhat confused in that he reported ascospore formation as well as the existence of haploid, diploid, triploid and tetraploid forms.

In France, the first mention of these yeasts in wines was made by BARRET, BIDAN and ANDRÉ (1955) who reported the occurrence of *Brettanomyces* species in the 'vins jaunes' from Arbois,

which had likewise shown acetification in the absence of bacterial infestation. This report was supplemented by an account from GALZY and RIOUX (1955) of these yeasts in the 'fleur' pellicles of wines from the 'Midi' region. Subsequently, DOMERCQ (1956), PEYNAUD and DOMERCQ (1956) and PEYNAUD (1956) described the occurrence of *Brettanomyces* in wines, musts and in the cellars of the Gironde area.

The occurrence of *Brettanomyces* in Italian wines was reported by VERONA (1951) and CASTELLI (personal communication).

On the other hand, their presence has not yet been reported in Californian wines, notwithstanding the fact that yeast turbidity is not uncommon in unfortified wines there. It is not known whether this is due to differences in climatic conditions or to differences in viticultural and oenological practices.

The above will suffice to show that *Brettanomyces* species constitute a hazard in wine making in that they may cause two types of failure in wine. As SCHANDERL and DRACZYNSKI (1952), BARRET *et al.* (1955), DOMERCQ (1956) and PEYNAUD and DOMERCQ (1956) found, excessive amounts of acetic acid may be formed. This phenomenon only occurs, as CUSTERS (1940) showed, when the yeasts have access to molecular oxygen, when alcohol is converted to acetic acid. If practicable, the rigorous exclusion of air will prevent such acetification. The second type of spoilage, which was reported by SCHANDERL and DRACZYNSKI (1952) and which is most prevalent in South Africa, is the formation of troublesome fine dusty yeast sediments.

As these yeasts are relatively insensitive to sulphur dioxide and capable of vigorous development in wines containing up to 13% alcohol, filtration, preferably through K7-K10 filter sheets appears at present to be the only method for treating wines infected with these organisms.

It is not unlikely that the increased incidence of yeast turbidities in South African wines during the last decade is due to an increase in the *Brettanomyces* incidence, since the incidence of the remaining significant species seems to be of the same magnitude as that reported, for instance, in France.

The question now arises as to the origin of the *Brettanomyces* species in wine. Their presence could hardly be accounted for by their adaptation to this milieu in recent years. On the other hand, studies of the yeast flora associated with grapes and musts

[in Italy (CASTELLI 1954, 1956), Sicily (CASTELLI 1954), Sardinia (VERONA 1951), Greece (VERONA, PICCI, MELAS-JOANNIDES and CARNI 1956), Spain (CASTELLI and IÑIGO LEAL 1957), Israel (CASTELLI 1954), Brazil (VERONA and ZARDETTO DE TOLEDO 1954), France (DOMERCQ 1956), North Africa (BERAUD 1937) and in California (MRAK and McLUNG 1940)] — have shown that these yeasts are not associated with the normal flora. DOMERCQ (1956) does, however, record their presence in two of thirty-three samples of red wine must from the Gironde. Accordingly, it may be concluded that these yeasts are associated rather with the cellars and cellar equipment, and cause infestation from such sources. This opinion seems supported by the observation of PEYNAUD (1956) that *Brettanomyces* species frequently occur in the mould mat developing on the walls and in the soil of damp cellars.

In comparing the percentage incidence of *Brettanomyces* obtained in this survey with that obtained by DOMERCQ (1956) for yeasts associated with bottled wines of the Gironde, it will be noted that the incidence in South African wines is about thirty-fold higher than that reported for the French wines. Furthermore, DOMERCQ (1956) only encountered these yeasts in one sample, a claret, of thirty samples of bottled red wines. These organisms are not listed by her as occurring in bottled white wines. Although the methods employed by the authors admittedly differ from those employed by DOMERCQ (1956), it still remains difficult to account for such discrepancy solely on the grounds of isolation techniques. Other factors are probably involved which give rise to this somewhat alarming incidence, particularly in view of the claims that there has been a definite increase in the yeast turbidity in South African wines.

2) *Saccharomyces oviformis*. — The occurrence of *Sacch. oviformis* in unfortified wines is not uncommon. SCHEFFER and MRAK (1951) in an examination of fourteen yeast cultures isolated from eighteen samples of cloudy dry white wines, found no less than six of these cultures to be *Sacch. oviformis*. DOMERCQ (1956) similarly reported thirty-five out of seventy wines to contain this species. Although the figure obtained by the authors (20%) is somewhat lower, it is nevertheless of the same magnitude.

SCHEFFER and MRAK (1951) in their investigations paid particular attention to the biochemical properties of this species in relation to its occurrence in dry wines. They showed that strains of this

species could tolerate high concentrations of alcohol (up to 18 vol. %) and sulphur dioxide (300 parts per million) in wines. The American authors also established that this yeast still developed in wines having a sugar concentration as low as 0.01%.

As DOMERCQ (1956) noted, this species tends to predominate during the final stages of the natural vinous fermentation by its ability to ferment residual sugar at relatively high concentrations of accumulated alcohol. Owing to its alcohol tolerance, this yeast causes re-fermentation of wines which were originally fermented by less alcohol-tolerant species, *e.g.* *Sacch. cerevisiae*. Because of its ability to bring about more complete fermentation, *Sacch. oviformis* is being increasingly used in wine making. PEYNAUD (1956), for example, advocates its application for the fermentation of both red and white dry wines. Its presence in such finished wines would be indicative of inadequate racking or fining.

3) *Saccharomyces acidifaciens*. — *Sacch. acidifaciens* appears as a rather frequent cause of turbidity in bottled wines. NICKERSON (1943) isolated this species from a sample of red wine which had shown considerable acetification in the absence of *Acetobacter* infection. DOMERCQ (1956) likewise isolated it from both red and white bottled wines from the Gironde. DOMERCQ (1956) and PEYNAUD (1956) pointed out that *Sacch. acidifaciens* resembled *Sacch. oviformis* in its ability to re-ferment unfortified wines of fairly high alcohol and moderate sulphur dioxide concentrations.

Although the percentage incidence of this species in South African dry wines, *i.e.*, 27%, is somewhat lower than that reported by DOMERCQ (1956) for the white wines of the Gironde (34%), they are of the same magnitude.

4) *Pichia membranaefaciens*. — This species, together with *Candida mycoderma*, appears to be common to natural unfinished wines and soon forms a pellicle on their surface, if unduly exposed to air. SCHEFFER and MRAK (1951) in their studies of yeast turbidities in Californian wines reported two strains of *Pichia alcoholophila* Klöcker 1912 — which LODDER and KREGER-VAN RIJ (1952) regard as identical to *Pichia membranaefaciens* — amongst fourteen yeast strains they had isolated.

The American authors established that development of these strains in wine was inhibited only at 15 vol. % alcohol and 300 ppm sulphur dioxide. Growth still took place in wines containing as little as 0.01% sugar. This species, as well as *Candida mycoderma*, is

characterised by a very weak fermentative dissimilation and relies for its growth predominantly on its oxidative metabolism. The presence of either of these organisms in wine suggests, therefore, its having been in contact with air for unduly long periods of time. The same holds for the strict oxidative yeasts reported in this survey, *i.e.*, *Cryptococcus diffluens* and *Cryptococcus laurentii*. It will be noted that the incidence reported by DOMERCQ (1956) for oxidative yeasts in bottled wines, or wines in bulk under storage, is extremely low, namely only one sample of white wine from fifty-two was found to contain *Candida mycoderma*.

5) *Saccharomyces cerevisiae*. — As *Saccharomyces cerevisiae* and its variety *ellipsoideus* are normally associated with a sound vinous fermentation, their presence in young dry wines is not abnormal. However, as *Sacch. cerevisiae* is not as alcohol-tolerant as, for instance, *Sacch. oviformis*, the cells do not survive in dry wines for any length of time. The occurrence of *Sacch. cerevisiae* in dry wines is generally due to incomplete clarification.

6) Other species. — Of the remaining species which showed an incidence of 5% or less, the following may be mentioned.

Saccharomyces cerevisiae var. *tetrasporus* closely resembling *Sacch. cerevisiae*, differs only in minor morphological and biochemical properties. As this organism has been isolated in several instances from soils of the Western Cape Province, it probably occurs in musts as well.

With the exception of *Candida mellini*, all other species listed have been isolated from vinous products elsewhere as well.

Attention may finally be drawn to the absence of *Saccharomyces ludwigii* in the wines examined in this survey. This contrasts rather sharply with the results obtained by DOMERCQ (1956), who reported it in eight of fifty-two white wines from the Gironde examined by her. According to DOMERCQ (1956) and PEYNAUD (1956), this species is particularly common in white wines with high sulphur dioxide content.

Reviewing the pattern of occurrence of the yeasts causing turbidities in South African wines, it appears that — with the exception of the high incidence of *Brettanomyces* species — the occurrence of the remaining species is quite similar to that reported in other wine producing countries. Further investigation is, however, necessary to account for the high incidence and apparent increase of the *Brettanomyces* infestation in South African wines.

Summary.

As a first step in the systematic study of the problem of yeast turbidities in South African bottled wines, a taxonomic survey of the causative organisms has been undertaken. A method of their isolation has been described. In this survey, sixty wine samples showing yeast hazes were examined. The samples included dry white, semi-sweet white, dry red, and medium sweet sparkling wines as well as pale dry sherries. At least fifteen yeast species were identified. Of these, only five have an incidence above 10%, namely, *Brettanomyces* species (50%), *Saccharomyces acidifaciens* (18%), *Saccharomyces oviformis* (20%), *Saccharomyces cerevisiae* (15%) and *Pichia membranaefaciens* (13%). The observed percentage incidence of *Sacch. acidifaciens*, *Sacch. oviformis*, *Sacch. cerevisiae* and *P. membranaefaciens* corresponds, within limits, to values reported for other wine producing localities. On the other hand, the observed incidence of the *Brettanomyces* species represents the highest value yet reported, being thirty-fold higher than that observed in wines from the Gironde area. This abnormally high incidence is most probably connected with the reported increase of yeast turbidities in South African wines during the last decade.

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INVESTIGATIONS INTO THE ANTIGENIC STRUCTURE OF THE REITER STRAIN OF *TREPONEMA PALLIDUM*

1. THE PRESENCE OF THE 'UBIQUITOUS LIPID' IN THE PROTEIN FRACTION

by

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(Received March 15, 1958).

INTRODUCTION.

In a previous paper, we studied the practical significance in the serodiagnosis of syphilis of a complement fixation test (CFT) in which a protein fraction derived from the Reiter strain of *Treponema pallidum* was used as an antigen (DE BRUIJN, 1957a). The discrepancy between the results obtained in the CFT with protein antigen, in the CFT with cardiolipin antigen and in the *Treponema pallidum* immobilization test, suggest the presence in syphilitic serum of at least three antibodies. It has been shown before, that absorption of syphilitic serum with protein antigen does not alter its reactivity with cardiolipin antigen (DE BRUIJN, 1956). The present paper is a study of the influence of absorption with cardiolipin antigen upon the serological pattern of the reaction between syphilitic serum and protein antigen. In this reaction, the protein antigen usually shows an optimum in the 1 : 80 dilution (DE BRUIJN, 1957a). Recently, starting with the 1 : 2.5 instead of with the 1 : 5 dilution, another optimum was observed at the lowest antigen dilution (DE BRUIJN, 1957b). These optima have been attributed to the presence in the protein fraction of a major and a minor component respectively. It has been suggested that the minor component is identical with a fraction obtained from protein antigen by ethanol-treatment. The serological and chemical properties of this fraction are the subjects of this paper.

MATERIALS AND METHODS.

Preparation of the protein antigen.

The protein antigen was prepared according to the directions given by D'ALESSANDRO and DARDANONI (1953), as previously described (DE BRUIJN, 1957*a*).

Preparation of the alcohol-soluble fraction of protein antigen.

To 10 ml of protein antigen, 27 ml absolute ethanol was added under continuous stirring, thus giving a concentration of 68% ethanol by weight. The mixture was kept in a 5°C. refrigerator overnight and centrifuged. The supernatant fluid was decanted and evaporated in vacuo at 30°C. until dry. The residue was either dissolved in 10 ml distilled water or extracted with 5 ml methanol.

Complement fixation test.

The CFT used was a highly standardized routine technique (DE BRUIJN, 1958). The serum was a pool of human syphilitic sera. Appropriate serum- and antigen controls were included in each test.

Absorption of cardiolipin antibody from syphilitic serum.

The serum to be absorbed was heated in a 56°C. waterbath for 30 minutes. According to the method of HARDY and NELL (1955), absorption was carried out with VDRL antigen (0.03% cardiolipin, 0.21% lecithin and 0.9% cholesterol in absolute ethanol) as follows: Into separate tubes, 10 ml 0.85% sodium chloride solution and 2 ml of the antigen were pipetted respectively. The saline was added rapidly to the antigen and mixed thoroughly by pouring from one tube into the other and back six times. The suspension was centrifuged and the supernatant fluid discarded. The precipitate was suspended in 2 ml of the serum to be absorbed, incubated in a 37°C. waterbath for one hour under occasional shaking and kept in a 5°C. refrigerator overnight. The tubes were centrifuged and the serum pipetted from the precipitate. Before testing it was reheated at 56°C. for 10 minutes. The serum, originally showing a titer of 1 : 32 in the CFT with cardiolipin antigen, was non-reactive in the 1 : 1 dilution after one absorption.

RESULTS AND DISCUSSION.

Both the original serum and the serum absorbed with cardiolipin antigen were subjected to titration with protein antigen (table 1) and to titration with the alcohol-soluble fraction of protein antigen, dissolved in distilled water (table 2).

TABLE 1.

Titration with protein antigen of the original serum (O) and of the serum absorbed with cardiolipin antigen (CA).

O serum dilutions	Antigen dilutions							
	1:1	1:2.5	1:5	1:10	1:20	1:40	1:80	1:160
1:5	++++	++++	++++	++++	++++	++++	++++	++
1:10	++	+	—	+	+++	++++	++++	++
1:20	+	—	—	—	—	++	++	+
1:40	—	—	—	—	—	—	—	—

CA serum dilutions	Antigen dilutions							
	1:1	1:2.5	1:5	1:10	1:20	1:40	1:80	1:160
1:5	+++	++	++++	++++	++++	++++	++++	++
1:10	—	—	—	—	++	++++	++++	++
1:20	—	—	—	—	—	+	++	+
1:40	—	—	—	—	—	—	—	—

TABLE 2.

Titration with the alcohol-soluble fraction of protein antigen of the original serum (O) and of the serum absorbed with cardiolipin antigen (CA).

O serum dilutions	Antigen dilutions							
	1:1	1:2.5	1:5	1:10	1:20	1:40	1:80	1:160
1:5	++++	++++	+++	+	—	—	—	—
1:10	+	+	—	—	—	—	—	—
1:20	—	—	—	—	—	—	—	—

CA serum dilutions	Antigen dilutions							
	1:1	1:2.5	1:5	1:10	1:20	1:40	1:80	1:160
1:5	—	—	—	—	—	—	—	—

As the optimum at the lowest antigen dilution disappeared with the removal of cardiolipin antibody (reagin) from the serum, the minor component of protein antigen appears to be serologically related to cardiolipin antigen. In the 1:80 dilution, the activity of this component is obviously diluted out, as the absorption does not affect this peak (table 1).

From table 2 it can be concluded that the major component of

protein antigen was precipitated by ethanol 68%, whereas the minor component remained unimpaired.

The phosphatidic nature of the minor component could be established by its precipitation from the aqueous 'solution' with an excess of acetone. Cardiolipin- and lecithin-like substances could be demonstrated in the methanolic extract by successive precipitation with barium- and cadmium chloride according to PANGBORN (1944).

Until now, the protein fraction has been considered to be devoid of the 'ubiquitous lipoidal' antigen (D'ALESSANDRO and DARDANONI, 1953). The evidence to the contrary does not diminish the value of the protein antigen in the serodiagnosis of syphilis, provided that it is used in a dilution in which only the major (protein) component is active.

Recently, when the stability of protein antigen stored under tropical conditions was studied, the lyophilized antigen was heated at 56°C. for up to 4 weeks. Whereas the major (protein) component underwent no appreciable loss of activity, the minor (lipoidal) component showed a marked decline.

Further investigations are in progress to determine to what extent the 'ubiquitous lipid' is associated with the protein.

Summary.

The presence has been demonstrated of the 'ubiquitous lipid' in the protein fraction of the Reiter strain of *Treponema pallidum*. As the lipid is serologically active only in low dilutions, no 'biologic false positive' results, due to its reaction with the reagent of the serum, are to be expected, provided that the protein antigen is applied in a sufficiently high dilution.

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A COMPARATIVE STUDY OF THE TRANSFORMATION OF GRAM-NEGATIVE RODS INTO "PROTOPLASTS" UNDER THE INFLUENCE OF PENICILLIN AND GLYCINE

by

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(Received June 11, 1958).

INTRODUCTION.

The bacteriolytic action of penicillin was first recorded by FLEMING (1929) during his pioneer's observations on a crude culture-filtrate from *Penicillium notatum*. It was found again, later on, with the purified antibiotic (see FLOREY *et al.* 1949).

The mechanism of this action was not quite clear (WELSCH, 1947c). Although crude or purified penicillin has a slow and weak lytic effect upon suspensions of resting staphylococci (WELSCH, 1947a), this is in no way comparable to that of, for instance, actinomycin (WELSCH, 1947b; GHUYSEN, 1957). On the other hand, growing cultures, submitted to the action of the drug, sometimes undergo lysis, but sometimes, do not. On the basis of these observations, it was more or less generally admitted that penicillin-bacteriolysis is a facultative and secondary phenomenon, following upon the release or activation of the autolytic system of the sensitive organism (TODD, 1945), when such a system is present (WELSCH, 1957a).

More recently, however, LEDERBERG (1956) has shown that gram-negative rods, such as *Escherichia coli* and other *Enterobacteriaceae*, are lysed when submitted to a high enough concentration of penicillin, although these organisms possess at most a weak autolytic system. He ascribes penicillin-lysis to an inhibition of the biosynthesis of the cell-wall (LEDERBERG, 1957; PARK and STROMINGER,

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1957; PARK, 1958), followed by the bursting of the naked bacterial cells in the usual culture-media which are hypotonic. In fact, if a stabilizer such as sucrose, originally used by WEIBULL (1953) to control lysozyme-induced bacteriolysis, is added to the menstruum, lysis does not occur but, instead, the micro-organisms are transformed into spherical bodies considered as protoplasts or protoplast-like elements. "Protoplast" formation from *E. coli*, *Shigella*, *Proteus* and *S. enteritidis* submitted to the action of penicillin was also reported (LIEBERMEISTER and KELLENBERGER, 1956; HAHN and CIAK, 1957; MICHAEL and BRAUN, 1958, WELSCH, 1958c; VADASZ and JUHASZ, 1955).

Penicillin being also known as the best inducer of the L-cycle (DIÉNÈS and WEINBERGER, 1951), LEDERBERG (1957) suggested that the "large body", found at the start of this cycle is, in fact, a protoplast.

Several instances of bacteriolysis were recently reported as resulting from a destruction, or lack of formation, of the bacterial cell-wall (see: WELSCH, 1957b, 1958a), in particular in the case of glycine-induced lysis (WELSCH, 1958b, c). This amino-acid behaves in many ways as penicillin. Thus, it has a bacteriolytic effect upon various micro-organisms (MACULLA and COWLES, 1948; GORDON, HALL and STICKLAND, 1949, 1951a, b, 1952, 1953, 1954; SALMON, 1952, 1957; SMOLIAR, 1952), although, in contradistinction with penicillin, it lyses resting (but not killed) suspensions as well as growing cultures. Glycine also induces morphological abnormalities (GORDON and GORDON, 1943, 1947; GORDON and HALL, 1950) comparable to those described in penicillin-treated cultures (see: FLOREY *et al.*, 1949). Among many chemicals tested, glycine was nearly the only one to compare with penicillin as a potent inducer of the L-cycle (DIÉNÈS and ZAMENICK, 1952). Finally, glycine was recently found capable of transforming *Escherichia coli* into "protoplasts" (WELSCH, 1958b, c) when acting in a medium supplemented with sucrose. Again, this phenomenon occurs with resting as well as with growing cells.

The present paper is devoted to a comparative study of the action of glycine and penicillin upon several gram-negative rods with the aim of finding out whether transformation into "protoplasts" by these agents occurs generally and through similar mechanisms, and whether differences are to be found in the response of various species and strains.

MATERIAL AND METHODS.

A. The organisms used in the present work, unless otherwise stated, were isolated from pathological samples submitted to the laboratory for routine analysis. According to the classification of Bergey's Manual, they include 25 strains representing 15 species, 10 genera and 3 families. They are:

a. Pseudomonadaceae:

1. *Pseudomonas aeruginosa*: three motile strains, all producing the typical pigment pyocyanin.

b. Rhizobiaceae:

2. *Chromobacterium janthinum*: one motile strain isolated from well-water.

c. Enterobacteriaceae:

3. *Escherichia coli*: two motile strains. They both have all the biochemical properties of the species. However, one ferments salicin early but is anaerogenic while the other ferments salicin slowly and is aerogenic.

4. *Escherichia freundii*: one motile collection strain, fitting into the Bethesda-Ballerup group (EDWARDS, WEST and BRUNER, 1948), possessing the Vi-antigen and labelled as *Salmonella ballerup* (KAUFFMANN, 1954; EDWARDS and EWING, 1955).

5. *Aerobacter aerogenes*: one non-motile strain.

6. *Klebsiella pneumoniae*: two strains respectively isolated from the upper respiratory tract of a child and of a chimpanzee (OSTERRIETH, 1958). The first one does not ferment dulcitol, while the second does.

7. *Paracolobactrum aerogenoides*: one non-motile strain isolated from the faeces of a chimpanzee (OSTERRIETH, 1958) which fits into MØLLER's (1954) proposed genus *Hafnia*.

8. *Serratia marcescens*: one motile strain isolated from tap-water.

9. *Proteus mirabilis*: three motile strains.

10. *Proteus inconstans*: two strains, both motile, fitting into KAUFFMANN's (1954) proposed genus *Providencia* and belonging to biochemical group I of EWING, TANNER and DENNARD (1955).

11. *Salmonella enteritidis*: one strain, motile, agglutinated by antisera IX and g, m.

12. *Salmonella typhosa*: three strains, all motile, agglutinated by antiserum d, but being respectively in phase V (Vi: +; IX:o), VW (Vi: +; IX: +) and W (Vi:o; IX: +) of KAUFFMANN (1954).

13. *Salmonella spec.*: one strain, motile, belonging to KAUFF-

MANN's group B, agglutinated by anti-serum IV, V, but not further identified since being in the non-specific phase 1, 2.

14. *Shigella flexneri*: two strains belonging respectively to EWING's (1949) sero-groups II and IV.

15. *Shigella sonnei*: one strain.

B. All cultures were made in Difco Brain-Heart Infusion or on Brain-Heart Infusion Agar, respectively called hereafter "broth" and "nutrient agar".

The bacteria for experimentation were obtained by scraping the 14-hour growth from a nutrient agar culture in a Roux' bottle and suspending in 10 ml broth. One ml of the bacterial suspension was then added to each solution (10 ml) to be tested, placed in a 50 ml Erlenmeyer flask. The flasks were incubated on a reciprocal shaker at *ca.* 30°C. during 6 hours and thereafter left standing on the laboratory bench. Samples were removed hourly during the first 6 hours and then after 8, 10 and 24 hours. They were microscopically examined with phase-contrast and dark field, using Heine's condensor from Leitz.

C. The action of penicillin (crystalline potassium salt of benzylpenicillin) was studied in broth (3 vol.) containing 10% sucrose (1 vol. of a 40% w/v aqueous solution). Following LEDERBERG (1956), the concentration of the antibiotic was initially chosen as 1.000 u/ml, but was raised or lowered as needed.

D. On the basis of WELSCH's observations (1958*b*, *c*), the action of glycine was studied, in broth diluted 1 in 4, under the following conditions: glycine M/2 alone and, respectively, with 4, 10 and 20% sucrose, glycine M with 10% sucrose. A control culture without glycine, but containing 10% sucrose, was set up in each case.

E. Serological investigations were made with Wellcome's diagnostic sera, using slide- and test-tube agglutination (followed by macroscopic and microscopic examination) according to KAUFFMANN's (1954) techniques but with live suspensions. In several cases, the technique of agglutinin-absorption was also used.

EXPERIMENTAL.

A. Stages of the transformation.

When bacterial suspensions, submitted to the action of a suitable concentration of penicillin in sucrose-broth, are frequently examined, one can generally observe several successive stages occurring

between the initial normal rod and the final typical sphere. This was particularly well seen in the case of *Salmonella typhosa* and *Escherichia freundii* (= *S. ballerup*) when treated with 10 u/ml in broth containing 10% sucrose.

The first abnormal forms to be seen after 2—3 hours were rods and even filaments. Next a swelling, sometimes two, exceptionally more, appeared either near the middle or at one end of the long forms. They enlarged, became spherical and, already at this stage, reminded one of the protoplasts. In a few cases, the enlargement could be seen as a laterally located bulge on one side of the filament. Later on, a large portion of the filamentous part disappeared and spherical bodies, bearing one or two rather short appendages, were seen. Finally, the appendages were lost and spherical protoplast-like bodies were left. It should be stressed that, in contradistinction with LEDERBERG and ST. CLAIR's statement (1958), active motility was often observed for some time in all these abnormal forms, including the "protoplasts", whenever they were derived from motile rods.

When the concentration of penicillin is too high, the transformation may occur so quickly that the sequence of events cannot be followed. This was generally the case also for glycine-treated suspensions. Then either a mixture of different abnormal forms or only spherical bodies, "protoplasts", were observed. On the contrary, in other cases, abnormal forms appeared but never "matured" to the "protoplast" stage. Sometimes, greatly swollen rods, biscuit- or spindle-shaped, were seen instead of or along with long forms (*Proteus*, *Klebsiella*, *Aerobacter*).

In what follows, the transformation will be called "complete" when at least some typical spheres were observed, and "incomplete" when one or several of the intermediary stages only were seen. The transformation will be called "partial" when a substantial proportion of the bacteria retained their normal rod-like shape, and "total" when nearly all the germs assumed an abnormal appearance.

Normal and abnormal forms have initially a sharp outline and appear as dark, highly-contrasted structures in phase-contrast microscopy. In the absence of sucrose, and sometimes even in its presence, they may change into very poorly contrasted structures, becoming thinner, sometimes mottled, with an ill-defined outline. Those elements, undergoing lysis, will be called "ghosts".

Under optimal conditions, the first abnormalities were visible

after 2—4 hours. If their appearance were delayed for more than 6 hours, the transformation will be called "slow".

B. Action of penicillin.

a. After incubation with 1.000 u/ml, twelve of the strains examined behaved as *E. coli* W 1364 previously studied by WELSCH (1958*b, c*). Within 4 hours, nearly 100% of the rods were converted into spherical bodies resembling protoplasts. In several instances, despite the presence of 10% sucrose, there was evidence of lysis after a few hours. It was extensive with the two *E. coli*, *Salmonella* spec. and the three *Shigella*, ghosts from spheres and granular debris being the only structures left after 24 hours. Lysis was slight with *S. enteritidis* and the three *Proteus mirabilis*. In the latter case, rod-shaped ghosts were seen in addition to protoplast-ghosts and granules. No signs of lysis were observed with *Paracolobactrum aerogenoides* and *Proteus inconstans* n° 1. The latter organism was the only one with which intermediary stages (long forms) were observed.

However, when the antibiotic concentration was lowered to 200 (the two *E. coli*, *P. inconstans* n° 1, *Paracolobactrum aerogenoides*), or 50 u/ml (the three *P. mirabilis*, the three *Shigella* and the two *Salmonella*) all types of intermediary forms were observed within 4 hours. The transformation remained partial and incomplete with *P. mirabilis* n° 1 and 2, partial and complete with *S. sonnei*, total and complete with the other micro-organisms, spherical bodies being the only elements left after 24 hours in the case of *E. coli* n° 2, *P. mirabilis* n° 3 and *Salmonella* spec.

With the organisms most sensitive to lysis, ghosts of normal rods and of all abnormal forms were seen more or less early.

All the organisms of this group grew well, from a small inoculum, in broth containing 10, in some cases 100 u/ml penicillin. None grew in the presence of 1.000 u/ml, except *P. inconstans* n° 1 which slowly developed under such conditions.

b. A second group comprises five organisms which, with 1.000 u/ml, underwent only a slow, incomplete and very partial transformation. Protoplast-like bodies were exceptional, but other abnormal forms, such as swollen rods (*Aerobacter aerogenes*, the two *Klebsiella*) and long forms, with and without swellings (the two *Klebsiella*, *Proteus inconstans* n° 2, *Serratia marcescens*), were more or less numerous.

However, raising the antibiotic level to 2.500 (*A. aerogenes*), 5.000 (the two *Klebsiella*, *P. inconstans* n° 2) or 10.000 u/ml (*S. marcescens*) resulted in a substantial increase in the number of spheres and other abnormal forms, amounting in all cases, except with *S. marcescens*, to a nearly total transformation. With the latter organism, a total transformation, with an early extensive lysis, was obtained when the penicillin level was raised to 50.000 u/ml. In that case, typical "protoplasts", their ghosts and ghosts of normal rods were observed.

With the exception of *A. aerogenes*, organisms of this group developed well in broth containing 1.000 u/ml, *P. inconstans* n° 2 and *S. marcescens* even growing, although slowly, in the presence of 10.000 u/ml.

c. A third group comprises organisms undergoing no transformation at all (*C. janthinum*) or only a slow, incomplete and very partial one (the three *P. aeruginosa*), even when the antibiotic concentration was 10.000 u/ml. When it was raised to 50.000 u/ml, the three *P. aeruginosa* lysed quickly, ghosts of normal rods only being seen while a few motile bacteria survived. *C. janthinum* retained its normal rod shape but early lost its motility.

All these organisms grew normally in broth containing 1.000 u/ml and slowly when the concentration was raised to 10.000 u/ml.

d. The fourth and last group includes the three *Salmonella typhosa* and *Escherichia freundii* (= *S. ballerup*). In sucrose broth medicated with 1.000 or 200 u/ml penicillin, suspensions of these organisms lysed almost completely within 3 hours. In addition to granular debris, a few spheres and corresponding ghosts could be observed, but the main forms were long rods, often with a large terminal or central swelling. Some of them gave a normally contrasted image, but very many appeared as ghosts.

With 50 u/ml, three of the strains lysed early, many normal rods turning into ghosts without changing their general shape. The fourth one (*S. typhosa* in phase VW) behaved approximately as in the presence of 10 u/ml.

When the antibiotic concentration was lowered to that level, there was no gross evidence of lysis. Long filamentous forms, with and without swellings, appeared early. Later on, spheres bearing one or two appendages, and, still later, typical "protoplasts" were dominant. After 24 hours, most of the spheres, but also a number of the other abnormal forms, had turned into poorly contrasted ghosts.

Organisms of this group grew poorly, or not at all (*S. typhosa* in phase W), in the presence of 10 u/ml penicillin.

C. Action of glycine.

a. All the organisms examined were transformed to some extent into "protoplasts" by glycine M/2, more or less rapid lysis ensuing in the absence of sucrose. Except for *S. marcescens* and the three *P. mirabilis*, glycine M was never more, and usually less effective as an inducer of the transformation.

"Protoplast"-formation was slow and remained partial with *E. coli* n° 1 and *S. marcescens*. It was partial with some of the *Proteus*. In this case we, at first, got the impression that sucrose inhibited the phenomenon. In fact, "protoplasts" formed in the presence of sugar are and remain very small, while those occurring in glycine alone swell and gain a much larger size. With all the other organisms, the transformation started within 6 hours, often much earlier, and was generally complete and total, or nearly so.

"Protoplasts" from the five *Proteus*, from *P. aerogenoides*, from the *Salmonella*, from *S. flexneri* II and from *S. sonnei* were fairly stabilized by 4% sucrose in glycine M/2; those of *P. aeruginosa* n° 1 by 10%; those of *P. aeruginosa* n° 2 and 3, of *E. coli* n° 2, of *Aerogenes* and of *S. flexneri* IV by 20% (or by 10% in glycine M). Even with the highest concentration of sugar, other organisms showed signs of lysis after a few hours: *C. janthinum*, *E. coli* n° 1, *E. freundii* and the two *Klebsiella*.

After prolonged incubation, a secondary growth of normal bacillary forms often occurred with the three *P. aeruginosa* and some of the *Proteus*.

Intermediary stages were much less often observed than in penicillin-treated suspensions. However, long forms and spheres with appendages were seen transitorily in the case of the three *S. typhosa*, lastingly with the three *P. aeruginosa*. Similar forms, but especially swollen rods, biscuit-shaped and spindle-shaped, were seen in the case of the five *Proteus*.

In lysing suspensions, with or without sucrose, small granules and ghosts from "protoplasts" were regularly observed, accompanied, in some instances, by ghosts from normal rods and, with suitable organisms, by ghosts from intermediary forms.

b. Broth containing glycine M/2, M/8 and M/32, but no sucrose,

was lightly inoculated with each one of the organisms under study. Samples from the tubes showing evidence of turbidity after respectively 6 and 24 hours were microscopically examined.

All the bacteria under study grew normally in broth containing glycine M/32 and M/8. In the lower concentration, ghosts from rods were observed, along with normal motile rods, in the case of the two *E. coli*, while abnormally long motile forms and their ghosts were seen, in addition, in the case of *E. freundii* and *S. typhosa* VW. In the higher concentration, normal motile rods were observed with the three *P. aeruginosa*, *S. marcescens* and the five *Proteus*. Spherical bodies and intermediary forms, together with their ghosts, were seen, either after 6 or 24 hours and in more or less large number, with the other organisms, the three *Shigella* excepted. The cultures of the latter showed normal rods after 6 hours which, thereafter, turned into ghosts without change of their general shape and without intermediary forms or "protoplasts" being seen.

In broth containing glycine M/2, *S. flexneri* II showed a slight turbidity after 6 hours. Upon microscopic examination, only typical "protoplasts" and their ghosts were seen, the proportion of the latter steadily increasing upon further incubation. A similar evolution, although slower, was noted with *S. marcescens*. On the contrary, the three *P. aeruginosa* and the five *Proteus* grew quite well, a mixture of normal motile rods, intermediary forms and "protoplasts", together with all the corresponding ghosts, being seen after 6 as well as after 24 hours.

c. A few experiments were undertaken with the hope of obtaining L-forms from glycine-induced protoplasts. Totally transformed suspensions of *E. coli*, *Shigella sonnei* and *Salmonella enteritidis* were serially diluted in normal broth and in sucrose broth. Suitable dilutions were then plated, by the pour-plate method, in nutrient agar, in semi-fluid nutrient agar and in the latter medium containing 50% calf serum. Approximately the same number of colonies appeared on the three media, whether inoculated with samples from broth or sucrose-broth. They were all, however, of the normal "bacterial" type and not a single L-colony was observed.

Totally transformed suspensions of *Klebsiella* and *Proteus* were inoculated on Difco P.P.L.O. agar supplemented with Difco ascitic fluid, but again no L-colonies were obtained.

d. The observation that intermediary stages and "protoplasts" from motile organisms, display a very active motility, sometimes

for many hours, led us to investigate the antigenic properties of the glycine-induced protoplasts.

Suspensions of *Salmonella enteritidis* (IX; g, m: by slide agglutination) and of *Salmonella* spec. (IV, V; 1, 2; by slide agglutination), both normal and totally transformed into "protoplasts" by glycine in 10% sucrose-containing broth, were submitted to the action of anti-sera: IX; IV, V; g, m; and 1, 2. Macroscopic observation demonstrated a specific H-agglutination of normal rods and corresponding "protoplasts" and a specific O-agglutination of normal rods which was doubtful for corresponding protoplasts. However, microscopic observation conclusively showed that protoplasts were in fact specifically agglutinated by the corresponding anti-O serum. In addition, the anti-serum-treated suspensions were centrifuged and their supernatants tested against normal bacteria. The results obtained showed that H and O agglutinins were specifically absorbed by normal rods and corresponding "protoplasts".

Similar experiments were made with *S. sonnei*, *S. flexneri* II and *S. flexneri* IV. Macroscopic observation in the case of the first organism, agglutinin-absorption for the other two, again showed that specific O-antigens were present in the protoplasts.

Lastly, experiments of the same kind were performed with *E. freundii* (Vi⁺) and the three representatives of *Salmonella typhosa*, all d and, respectively, V, VW and W by slide agglutination. Macroscopic observation in the case of H- and Vi-agglutination, agglutinin-absorption for the O-agglutination, confirmed that H and O antigens are retained by the protoplasts and showed, in addition, that this is also true for the Vi-antigen.

DISCUSSION.

Transformation of gram-negative rods into "protoplasts" under the influence of either penicillin or glycine appears to be a rather general phenomenon. Among the 25 organisms studied, penicillin failed to transform only *C. janthinum* and the three *P. aeruginosa*, inducing however morphological abnormalities of the latter which can be considered as intermediary stages. Glycine, on the other hand, induced "protoplasts" in all the organisms examined, the transformation remaining very partial, however, with some of the *P. mirabilis* (see Table 1).

In the case of penicillin, the rate, completeness and extent of the transformation, as well as the concentration of antibiotic needed

TABLE 1.
Morphological changes of gram-negative rods in:

Micro-organisms	Sucrose-broth with								Broth with Glycine		
	Penicillin u/ml						Glycine				
	10	50	200	1000	2500 to 10,000	50,000	0.5 M	M	M/32	M/8	M/2
<i>E. freundii</i>	C(s)Tl	L	L	L	—	—	CsTl	—	Il	CL	—
<i>S. typhosa</i> V	C(s)Tl	L	L	L	—	—	C(s)T	—	O	CL	—
<i>S. typhosa</i> VW	C(s)Tl	C(s)Tl	L	L	—	—	C(s)T	—	Il	CL	—
<i>S. typhosa</i> W	C(s)Tl	L	L	L	—	—	C(s)T	—	O	CL	—
<i>Salmonella</i> spec.	—	C(s)T	—	CsTL	—	—	CsT	—	O	CL	—
<i>S. enteritidis</i>	—	CT	—	CsTl	—	—	CsT	—	O	CL	—
<i>S. sonnei</i>	—	CP	—	CsTL	—	—	CsT	—	O	L	—
<i>S. flexneri</i> II	—	CT	—	CsTL	—	—	CsT	—	O	L	CsL
<i>S. flexneri</i> IV	—	CT	—	CsTL	—	—	CsT	—	O	L	—
<i>E. coli</i> 1	—	—	CT	CsTL	—	—	SCPl	—	l	CL	—
<i>E. coli</i> 2	—	—	C(s)T	CsTL	—	—	CsT	—	l	CL	—
<i>P. aerogenoides</i>	—	—	CP	CsT	—	—	CsT	—	O	CL	—
<i>P. mirabilis</i> 1	—	lP	—	CsTl	—	—	SCp	CT	O	O	Cl
<i>P. mirabilis</i> 2	—	lP	—	CsTl	—	—	SCp	CP	O	O	Cl
<i>P. mirabilis</i> 3	—	C(s)T	—	CsTl	—	—	CP	CP	O	O	Cl
<i>P. inconstans</i> 1	—	—	CT	C(s)T	—	—	CP	CP	O	O	Cl
<i>P. inconstans</i> 2	—	—	—	Slp	CT	—	C(s)T	—	O	O	Cl
<i>A. aerogenes</i>	—	—	—	Slp	CT	—	CsT	—	O	CL	—
<i>K. pneumoniae</i> 1	—	—	—	Slp	CT	—	CsTl	—	O	CL	—
<i>K. pneumoniae</i> 2	—	—	—	Slp	CT	—	CsTl	—	O	CL	—
<i>S. marcescens</i>	—	—	—	Slp	CP	CTL	SCP	SCP	O	O	CsL
<i>P. aeruginosa</i> 1	—	—	—	tr	Slp	L	CT	—	O	O	Cl
<i>P. aeruginosa</i> 2	—	—	—	tr	Slp	L	CT	—	O	O	Cl
<i>P. aeruginosa</i> 3	—	—	—	tr	Slp	IT	CT	—	O	O	Cl
<i>C. janthinum</i>	—	—	—	O	O	O	CsTl	—	O	CL	—

Cs: transformation into "protoplasts" without intermediary stages being seen.

C(s): intermediary stages seen but "protoplasts" finally predominant.

C: intermediary stages persisting together with "protoplasts".

I: transformation incomplete ("protoplast" stage not seen).

T: transformation is total.

P: transformation is partial; p: very partial; tr: only traces.

S: transformation is slow.

L: important lysis; l: slight lysis.

O: no morphological change observed.

—: not tested.

to induce it, vary from one organism to the other and are roughly related to their susceptibility to the bacteriostatic action of the drug. In the case of glycine, where the range of active concentrations is much narrower, the rate and intensity of the transformation vary widely according to the strain under consideration and are also roughly related to the minimal growth-inhibiting concentration of the amino acid.

The stability of the glycine-induced "protoplasts" is also quite variable. In some cases, lysis is evident even when 20% sucrose is present in the medium, while, in some others, spherical bodies are stable for many hours in sugar-less broth. This suggests that such bodies are not quite identical with true "protoplasts" (SPOONER and STOCKER,

1956). Instead of being truly naked cells, they may retain at least some part of the cell-wall which would be functionally impaired but not altogether absent. At any rate, the fact that "protoplasts" and other abnormal forms can exhibit motility for some time and retain the flagellar antigens, shows that the action of glycine on the external bacterial structures is highly specific.

No studies were undertaken about the stability of penicillin-induced "protoplasts" from the present organisms, but, according to WELSCH's observations (1958c) with *E. coli* W 1364, the same remarks probably apply to them also. It should be noted that, in our experiments, no Mg^{++} or Ca^{++} were added to the medium.

If one compares the stability of respectively penicillin- and glycine-induced "protoplasts" of some of the organisms, for instance *S. typhosa*, it appears that the former are often more fragile. This is very likely due to two factors already pointed out by WELSCH (1958c), namely, that glycine, at the high concentration used, has some stabilizing effect, and that penicillin, at the proper concentration, exerts a direct lytic action upon "protoplasts".

The fact that organisms highly sensitive to the "protoplast"-inducing effect of glycine (*C. janthinum*, *P. aeruginosa*) are very resistant to that of penicillin, and *vice-versa* (several of the *Proteus*), is very likely an indication that the mode of action of the two agents are at least not entirely identical.

With both agents, under suitable conditions, and more easily with penicillin than with glycine, it can be seen that the transformation into spheres does not occur suddenly as with lysozyme (WEIBULL, 1953; ZINDER and ARNDT, 1956; REPASKE, 1956), when the cell-wall is peeled off the cytoplasmic membrane at once. On the contrary, it is often preceded by the appearance either of long even filamentous forms, showing later on one or several localized swellings, or of swollen, biscuit- and spindle-shaped elements. These abnormal forms, early recognized in penicillin- as well as in glycine-medicated cultures, especially on agar-media, were interpreted as evidence for an inhibition of cell division without hindering of growth or of nuclear multiplication (see: WELSCH, NIHOUL and DEMELENNE-JAMINON, 1948). They are easily interpreted according to current ideas and, in fact, this was done as early as 1946 by DUGUID. Thus, inhibition of cell-division and formation of giant forms may reflect the incapacity of the organism to synthesize its cross-walls. Swellings appearing next on the long and filamentous

forms very likely indicate the spots where absence of the lateral cell-wall synthesis begins. The cytoplasm, enclosed in its semi-permeable membrane, bulges through these points of lowered resistance assuming progressively a spherical shape. The cell then appears as a spherical body bearing one or two appendages which are the remnants of its empty cell-wall. Finally, leaving such fragments behind, it takes the shape of a typical "protoplast".

The whole process, when it can be followed in detail, is quite reminiscent of the "large body" formation as described by DIÉNÈS (1946), SCHELLENBERG (1954), BARTMAN and HÖPKEN (1956), RADLER and RIPPEL-BALDES (1956), whose observations, however, are not in perfect agreement with those of PULVERTAFT (1953), STEMPEIN (1955) and TAUBENECK (1955).

Glycine-induced "protoplasts", in general, are smaller than the corresponding ones obtained through the action of penicillin. This is to be expected since, with the amino acid, long forms are more rarely produced as a preliminary step to the transformation into spherical bodies. The size of the "protoplasts", in agreement with WELSCH's previous observations (1958c), also varies according to the sugar concentration used, it being smaller as the latter increases.

If the presence, in lysing suspensions, of ghosts from "protoplasts" is in good agreement with the idea that lysis may result from the osmotic destruction of such structures, the general observation of ghosts from normal rods, from long forms and filaments, with and without swellings, from swollen biscuit- and spindle-shaped bacteria, indicates that bacteriolysis can well affect those elements without their going through the "protoplast" stage. It therefore follows that the mechanism of penicillin-lysis proposed by LEDERBERG is probably not the only one to operate. The participation of the bacterial autolytic system or of an antibiotic-induced lytic enzyme (PRESTIDGE and PARDEE, 1957) must remain open. Criticism of the theory was also forwarded by GALE (1958) on entirely different grounds. In the case of glycine-bacteriolysis, WELSCH (1958c) has already pointed out several reasons for believing that "protoplast" formation is probably not the unique mechanism underlying the phenomenon.

Complete identity of the "protoplast" with the large body initiating the L-cycle remains an unsettled matter although, admittedly, L-forms and pleuropneumonia-like organisms, as well as protoplasts, seem to owe their peculiarities to the lack of a cell-

wall (KANDLER, ZEHENDER and MÜLLER, 1956; SHARP, HIJMAN and DIÉNÈS, 1957; WEIBULL, 1958), the absence of which, probably, explains their resistance to penicillin (WARD, MADOFF and DIÉNÈS, 1958).

As already reported for the stable L-forms of several bacteria (TULASNE, 1951; DIÉNÈS and WEINBERGER, 1951; SHARP, HIJMAN and DIÉNÈS, 1957) and for penicillin-induced "protoplasts" (LEDERBERG and ST. CLAIR, 1958), we have shown that glycine-induced "protoplasts" retain antigenic constituents believed to be rather superficially located, such as H, O and Vi-antigens, which, therefore, appear to have no relation with the cell-wall, or, at least, with those parts of the cell-wall which are the target of the "protoplasts"-inducer.

LEDERBERG and ST. CLAIR, in a paper which was received when the present one was already in manuscript, report the direct observation of L-colony formation from *E. coli* in a special agar medium containing sucrose and penicillin. The first abnormality to be seen was the rounding off of the rods into structures which, although less regular than typical protoplasts as observed in penicillin-sucrose broth, were considered as identical with them. It does not clearly appear whether the authors made similar observations when typical, preformed, "protoplasts" were embedded into the agar-medium but this was done by LANDMAN, ALTENBERN and GINOZA (1958). At any rate, it seems to us that a convincing proof of the complete identity of protoplast and large body would be the obtaining of L-colonies from preformed protoplasts in a medium devoid of any L-cycle inducer. Our attempts to give such a proof have unfortunately completely failed. However, the above mentioned paper of LEDERBERG and ST. CLAIR (1958) reports observations which greatly enforce the idea of identity between the two elements under discussion. A diaminopimelic acid-less mutant of *E. coli*, which lyses when grown in the presence of sub-optimal amounts of the growth-factor (BAUMAN and DAVIS, 1957; MEADOW, HOARE and WORK, 1957; RHULAND, 1957), is quantitatively converted into "protoplasts" when suspended in sucrose-broth and develops, but exclusively in the L-form, when inoculated into sucrose-agar. In this case, no external inducer is needed since the incentive for the transformation is to be found in an inherent metabolic defect of the strain, namely its incapacity for synthesizing DAP, a substance preferentially located in the cell-wall of which it

is an important constituent (WORK, 1957). In this connection, it may be reminded that, probably as a result of as yet unknown types of unbalanced growth (COHEN and BARNER, 1954), protoplast-like formations sometimes occur spontaneously in old cultures (SINKOVICS, 1957).

Conclusions and Summary.

Twenty five strains of gram-negative rods representing 15 different species were submitted to the action of either penicillin or glycine in normal or sucrose-enriched broth. Transformation into "protoplasts" under the influence of a suitable concentration of penicillin was obtained with nearly all of them, *Chromobacterium janthinum* and *Pseudomonas aeruginosa* being the only exceptions. Under the influence of glycine M/2 or M, "protoplasts" were obtained from all the organisms tested, but the transformation was slow and poor with *Proteus mirabilis*.

These differences in the range of activity of the two substances studied suggest that they do not act through strictly identical mechanisms.

Penicillin-induced protoplasts were generally more fragile than glycine-induced ones, probably on account of a stabilizing effect of glycine at high concentration and of a lytic action of penicillin. Wide differences of stability of the "protoplasts" were also noted from one strain to another.

The transformation was sometimes rather sudden but, especially with penicillin, can be progressive, intermediary stages, such as long and filamentous forms, with and without swellings, swollen rods, biscuit- and spindle-shaped, being seen when an appropriate concentration of the inducer is used.

In lysing cultures, ghosts from normal rods and from intermediary forms were generally seen in addition to "protoplasts" and their ghosts. This indicates that penicillin- and glycine-induced lysis can occur without the bacteria going through the stage of osmotically fragile protoplasts. The possible role of the autolytic system or of antibiotic-induced lytic enzymes therefore remains an open question.

Intermediary forms and protoplasts derived from motile rods retained their motility for some time. Glycine-induced "protoplasts" retained the H-, O- and Vi-antigens of the bacteria from which they were derived.

The identity of protoplasts with large bodies is discussed. It is pointed out that attempts to obtain L-colonies from protoplasts pre-formed in glycine by seeding them in agar media containing no known inducer of the L-cycle were unsuccessful.

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ON THE CELL WALL COMPOSITION OF *SACCHAROMYCOPSIS GUTTULATA*

by

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(Received July 31, 1958).

Considerable significance is attached to the cell wall composition in studying the relationship between different groups of microorganisms (1, 3, 4). In view of the fact that the taxonomic position of the genus *Saccharomycopsis* has not been clearly established in the past and at one time this monospecific genus was included in *Saccharomyces*, it was felt that an analysis of the cell wall composition of *Saccharomycopsis guttulata* might throw additional light on its relationship to *Saccharomyces*. SHIFRINE and PHAFF have recently described a method for isolating this yeast, and studied its morphology, physiology, and life cycle (12), as well as some of its nutritional requirements (11).

METHODS.

Total carbohydrates were determined by the anthrone method as modified by MILLER and PHAFF(8). Total nitrogen was determined after digestion of the sample by direct nesslerization following the method described by JOHNSON (6). Dry weight determinations were made in small aluminum moisture dishes. Drying was done in vacuum at 70°C. for 8 to 12 hours (constant weight). Chromatography (descending) was done on Whatman number one filter paper. Sugars were separated using isopropanol: ethanol (95%): water (7:2:1). The developing time was 24 hours. Silver nitrate spray developed by TREVELYAN was used for the detection of spots. Also used were triphenyltetrazolium chloride (for reducing sugars) and bromophenol blue (to detect organic acids). Two dimensional paper chromatography on Whatman number one paper was used

for the separation of amino acids. Solvents employed were phenol: water (4:2) and butanol: acetic acid: water (4:1:5), for the two consecutive runs. Ninhydrin spray was used to detect the spots. For details on chromatography, see BLOCK *et al.* (2). Fractionation of cell walls was done as described in detail by MILLER and PHAFF (8).

RESULTS.

Growing of the cells. *S. guttulata* was grown in standing cultures for one or two days at 37°C. in Fernbach flasks (capacity 31) containing 200 ml of yeast autolysate (10% v/v)-Proteose Peptone (Difco, 1% w/v) — glucose (2% w/v) (12). Use of more medium per flask reduced the cell density markedly. The period of growth was limited to two days to prevent excessive lysis of the cells (12). The cells were centrifuged, washed three times with water and finally suspended in water to give a count of 7×10^8 cells/ml.

Rupturing of the cells by sonic oscillation. A Raytheon oscillator (magnetostriction type), operating at 10 kilocycles, 250 watts, using maximum plate current (1.30—1.35 amperes) was used to disrupt the cells. Fifty ml of the suspension was added to the transducer of the oscillator. The gas phase was hydrogen and the temperature about 0°C. The decrease of the direct count of remaining whole cells (by phase microscopy) and change in turbidity are plotted as a function of time of oscillation in Fig. 1. Although sonication for 30 minutes ruptures over 95 per cent of the cells, large fragments result. On continued sonication, e.g. 60 minutes, these large fragments were further broken to smaller fragments, which are easier to purify. The time required to disrupt 50 per cent of the cells of *S. guttulata* was about 6 minutes. MILLER and PHAFF (8) showed that the half life of cells of *Hanseniaspora uvarum* is also *ca.* 6 minutes, but cells of *S. cerevisiae* required 32 minutes for 50 per cent breakage. Thus bakers yeast is much more resistant to sonication than is *S. guttulata*.

Purification of the cell walls. The sonicate was thrice centrifuged at high speed and resuspended in distilled water. Then 10 ml of the suspension were placed in a 12 ml conical centrifuge tube and centrifuged at 225 RPM in a clinical International centrifuge for 10 minutes. The bottom layer was cream colored and consisted mainly of whole cells. The upper layer was white and was

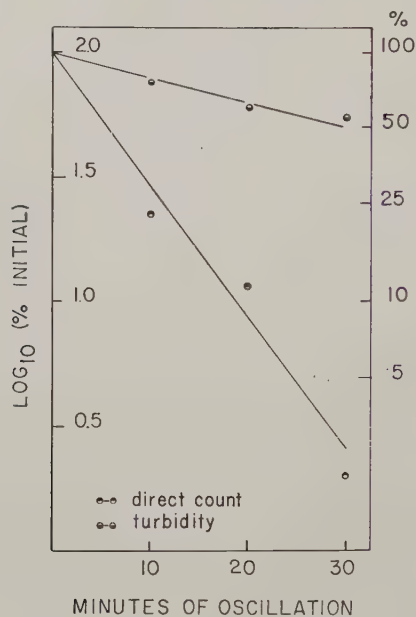


Fig. 1. Changes in the turbidity and direct count of whole cells of *S. guttulata* as a function of the time of sonic oscillation.

made up largely of cell walls. The supernatant liquid was discarded and the white upper layer was carefully resuspended with the aid of a glass rod. This suspension was removed with a pipet from the remaining sediment, which was discarded. This operation was repeated about 20 times, giving a preparation containing very few whole cells or granules. The yield of cell walls from whole cells was about five per cent (wet weight basis of both) after high speed centrifugation.

Composition of the cell walls. First, the amounts of total carbohydrate and protein present in the cell walls were determined and are given in Table 1. Directly determined figures

TABLE 1.

Composition of isolated cell walls of *S. guttulata*. Data for *S. cerevisiae*, obtained from the literature, are given for comparison.

	Total carbohydrates (expressed as glucose)	Protein (N \times 6.25)	Chitin (expressed as hexosamine)
<i>S. guttulata</i>	43.7	39.6	trace
<i>S. cerevisiae</i> (8)	82.8	6.5	trace
<i>S. cerevisiae</i> (5)	84.4	6.7	trace

(by the use of anthrone reagent) of other investigators, relating to *S. cerevisiae*, are given for comparison. Indirectly obtained values for total carbohydrate content of *S. cerevisiae* by ROELOFSEN (10) and by NORTHCOTE and HORNE (9) are lower (68% and 60%, respectively). The last authors, as pointed out by ROELOFSEN (10), disregarded the amount of glucan extracted by alkali along with the mannan. A comparison of directly determined values for the carbohydrate and protein content of *S. guttulata* and *S. cerevisiae* shows that the walls of the former species contain about half as much carbohydrate and six times as much protein as the latter species.

Next the cell walls of *S. guttulata* were hydrolyzed in 2N HCl for 30 minutes at 120°C. in sealed glass tubes. A similarly prepared cell wall preparation of *S. cerevisiae* was run for comparison. The hydrolysates were evaporated to dryness in vacuum over KOH pellets, taken up in a small amount of water and chromatographed on paper. The bakers yeast preparation showed strong spots corresponding to glucose and mannose and a very weak spot corresponding to glucosamine. The cell wall hydrolysate of *S. guttulata* showed five spots: a large spot corresponding to glucose (I), two very small and weak spots corresponding to mannose (II), and glucosamine (III), a fast-moving strong spot (of about equal intensity as glucose) with an R_{gluc} of about 1.41 (IV) and a slow moving rather weak spot with an $R_{gluc} = 0.55$ (V). By using variable amounts of glucose and mannose as markers, it was estimated that the glucose/mannose ratio was at least 4:1. Spot No. V may represent an incompletely hydrolyzed fraction of the carbohydrate. It is noteworthy that this spot was never observed with cell wall hydrolysates of bakers yeast, even though the hydrolysis was carried out under identical conditions. The fast moving spot did not show up with a ninhydrin spray and did not appear to have acidic properties. It did not correspond to xylose, arabinose, ribose, furfural, hydroxyfurfural or acetylglucosamine. It has a reducing group as shown by spraying with triphenyltetrazolium chloride. Its identity has not been investigated further.

The amino acid content was determined after hydrolysis with 6N HCl. The hydrolysate contained cysteic acid, aspartic acid, glutamic acid, serine, glycine, tyrosine, threonine, arginine, alanine, valine and/or methionine, leucine and/or isoleucine. The last four amino acids are listed in pairs since they do not separate well on

paper. All the amino acids found are also present in the walls of *S. cerevisiae* (5). However, bakers yeast contains in addition phenylalanine, lysine and an unidentified amino acid (5) which were absent in the walls of *S. guttulata*.

Next a cell wall preparation of *S. guttulata* was fractionated by the procedure described in detail by MILLER and PHAFF (8). The results are given in Table 2.

TABLE 2.

Fractionation of a cell wall preparation of *S. guttulata*. For details of the procedure see reference 8.

Fraction	Carbohydrate content	Protein content
Original cell wall material 36.25 mg	15.48 mg	14.35 mg
Hot 3% NaOH extract	4.4 mg	14.66 mg
Washed residue after alkali extraction	5.0 mg	none
Deficit (fraction not accounted for)	6.44 mg	—

The chromatographic analysis of the original cell material has been described above. The alkali extract contained all the protein and about one quarter of the carbohydrate fraction. Chromatography of its hydrolysate produced a strong spot for glucose and a weak spot for mannose. The residue after alkali extraction showed strong spots for glucose and the previously designated spot No. IV (about equal intensity). A small amount of glucosamine and of the material designated as spot No. V were also present. Mannose was absent. It will be noted that a significant amount of the carbohydrate fraction is unaccounted for. Possibly a part of this is lost during the various manipulations, such as washing and centrifuging of the alkali insoluble residue with dilute acetic acid and water. However, another part appears to be destroyed by heating with 3% alkali.

Part of the carbohydrates of purified cell walls of bakers yeast also appears to be alkali labile. No loss in carbohydrate content was noted (by the anthrone method) when bakers yeast cell walls were treated with 3 per cent KOH for one hour at room temperature. However, in a boiling water bath, between 10 and 20 per cent of the carbohydrates were destroyed, depending upon the experimental conditions. A sample of pure glycogen, on the other hand, appeared to be completely stable upon treatment with hot alkali.

DISCUSSION.

Isolated washed cell walls of the budding yeast *Saccharomycopsis guttulata* have an unusually large protein content (ca. 39.6%) and a low carbohydrate content (43.7%) as determined by the anthrone reagent. Whereas bakers yeast contains about equal amounts of glucan and mannan, a trace of chitin, and, according to most investigators, about 6 to 7 per cent protein, the walls of *S. guttulata* have a very different composition. The carbohydrate fraction, after hydrolysis and chromatography, shows about equal amounts of glucose and an unknown reducing compound, whereas mannose and glucosamine are present in low concentrations. The glucosamine content appears to be comparable to that present in *S. cerevisiae*, but the mannose content is much lower than that in bakers yeast. Mannose thus seems to be replaced by an as yet unidentified sugar or reducing compound. KREGER (7) also stated that in mannan deficient species, the mannan is mainly replaced by as yet unknown substances, although in some yeasts it is partly replaced by an increased chitin content. It is also noteworthy that under identical conditions of acid hydrolysis, the carbohydrates of the cell walls of bakers yeast are hydrolyzed to the monomer stage, but those of *S. guttulata* invariably produce a spot on chromatograms corresponding to a di- or trisaccharide. Fractionation of *S. guttulata* cell walls with hot 3% alkali resulted in a 40% loss of the carbohydrate fraction. A similar calculation of the values given by MILLER and PHAFF (8) for the walls of *Hanseniaspora uvarum* indicates a loss of about 12 per cent. Until enzymatic methods are available for the fractionation and analysis of cell walls, the currently used methods based on treatment with acid and alkali must be considered only approximately quantitative. All the amino acids present in the protein of the cell walls of *S. guttulata* have been reported in the cell walls of bakers yeast by FALCONE and NICKERSON (5). However, the walls of *S. cerevisiae* also contained phenylalanine, lysine and an unidentified amino acid, that were not detected in *S. guttulata*. There does not seem to be a relationship between the cell wall composition and rate of breakage due to sonic oscillation. *S. guttulata* and *Hanseniaspora uvarum* exhibit a much more rapid rate of cell rupture than *S. cerevisiae*, although the cell wall composition of *S. cerevisiae* and *H. uvarum* were shown to be similar (8). However, the unusual composition of the cell walls of *S. guttulata* may be

responsible for the rapid spontaneous lysis commonly observed in cultures of this yeast (12). Our findings tend to confirm previous conclusions (12) that *Saccharomycopsis* is a valid genus, which differs from *Saccharomyces* in many respects.

S u m m a r y.

Cells of *Saccharomycopsis guttulata* were ruptured by sonic oscillation and the resulting cell walls were purified by washing and centrifugation. The walls contained 43.7% carbohydrate (expressed as glucose), 39.6% protein and a trace of chitin. Paper chromatography of hydrolyzed cell walls showed that glucose and an unknown reducing compound make up the bulk of the carbohydrate fraction. Mannose and glucosamine were present in small amounts. The cell wall composition of *S. guttulata* appears to differ considerably from that of *S. cerevisiae*.

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STUDIES ON THE METABOLISM OF *ACETOBACTER PEROXYDANS*

PART I. — GENERAL PROPERTIES AND TAXONOMIC POSITION OF THE SPECIES

by

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(Received September 12, 1958).

INTRODUCTION.

Acetobacter peroxydans is an unusual bacterium which possesses several exceptional properties. It is classified amongst the acetic acid bacteria although its taxonomic position is not definite. In contrast to the other species of this genus, which are common inhabitants of spoiled beer and wine and in plant materials undergoing alcoholic fermentation, *A. peroxydans* is isolated from ditch water and sewage ²⁾ (VISSER 'T HOOFT, 1925; FRATEUR, 1950). VISSER 'T HOOFT isolated one strain from wine, FRATEUR (personal communication) isolated several strains from sugarbeet pulp.

¹⁾ Associé du Fonds National de la Recherche Scientifique (Belgique).

²⁾ Bergey's Manual of Determinative Bacteriology, Sixth Edition (1948), states that *A. peroxydans* may be isolated "From hydrogen peroxide solutions". VISSER 'T HOOFT has isolated three strains from ditch water and one from wine but never from H₂O₂ solutions. A possible explanation for this error could be given by a misinterpretation of the Dutch sentence of his thesis (p. 98-99). The translation reads: "*As it has already only just been mentioned in § 2 of this Chapter, I made use several times of a hydrogen peroxide solution in the isolation of acetic acid bacteria*, to distinguish with its aid colonies of the wanted acetic acid bacteria from colonies of lactic acid bacteria, which are sometimes found besides the former bacteria in the enrichment cultures" (italics are ours). If only this part of the sentence, which we put in italics, is considered, then the origin of the statement in Bergey's manual is explained. It is clear however that the hydrogen peroxide solution was used for the catalase test on the Petri plate, but not as a source for isolation.

A. peroxydans and *A. paradoxum* do not possess catalase, whereas all the other species do. Catalase is very widely distributed in the microbial world and is present in all aerobic bacteria, except in the micro-aerophilic lactic acid bacteria (genus *Lactobacillus*). These cells do not oxidize glucose, when grown on ethanol as main carbon source in yeast water (WIELAND and PISTOR, 1938) or in a synthetic medium (TANENBAUM, 1956). It would be of interest to establish whether they are able to grow on and to consume glucose at all, since the lack of this capacity would be really exceptional for a member of this genus.

Many strains are able to oxidize molecular hydrogen (VISSER 't HOOFT, 1925; WIELAND and PISTOR, 1936; ATKINSON, 1956; TANENBAUM, 1956a). This property has never been observed for other *Acetobacter* species. In view of these aberrant properties of *A. peroxydans*, as compared with the other members of this genus, a more extensive reinvestigation of this species seems to be necessary. In fact, the previously mentioned abnormalities do cast some doubt on the value of its present taxonomic position in the *Acetobacter* genus. A better knowledge of its characteristics might be helpful in determining its exact taxonomic position and phylogenetic relationships.

The incomplete information on *A. peroxydans* is the probable reason that it has been omitted from Bergey's Manual, Seventh Edition (1957). In this paper we wish to present a systematic study of the physiological and biochemical properties of several strains, which are preserved in different collections and bear the name of *Acetobacter peroxydans*.

EXPERIMENTAL AND RESULTS.

1. Bacteria used.

It is desired to extend here our thanks to the laboratories from which different strains were obtained. Strain 1 and 3 were obtained from the Laboratory for Microbiology, Technological University, Delft, Holland, through the courtesy of Dr LA RIVIÈRE. Strain 838 was purchased from the American Type Culture Collection. Through Mr Ira Mehlman, we obtained a strain from the collection of the Department of Food Technology, Davis, California, which we called „Davis". Strains 8618 and 8138 were presented to us by the curator of the National Collection of Industrial Bacteria,

Teddington, England. The behaviour of strain 8138 was completely different to that of the others. It will be shown that it was not a strain of *A. peroxydans* and for that reason will only cursorily be mentioned.

As soon as the bacteria were obtained, they were examined, plated out several times on beer-12% gelatine and incubated for 1–2 weeks at 20° C. Pure cultures were maintained on the same medium, stored in a dust-free closet at 18–20° C. and transferred to fresh medium every two or three weeks.

2. Aspect of the slants on beer-gelatine at 20° C.

S t r a i n s 1, 3, 838 and 8618. After two days of incubation the streak was weakly, but distinctly visible and nearly colourless. After one week there was good growth. The form of the culture was slightly echinulate, sometimes beaded, the luster was glistening and the consistency butyrous. The colour of the bacteria was white for strain 3 and pale brown for all the others. The medium remained unchanged. The bacteria are rod shaped, 2 — 3 μ long and about 0,5 μ in width. There are many diplobacters, which are very motile during the first few minutes on the slide, as has been described by FRATEUR (1950) and they are uniformly Gram negative. No involution forms were present in cultures up to one month old. Strain 3 differs from the others in that the cells are slightly larger.

S t r a i n Davis. They grow faster, formed a thicker pellicle and one week was required for good growth. The form was filiform, the luster dull, the consistency byturous. The colour was pale brown and the medium remained unchanged. The bacteria are short, thick, very motile rods (possibly peritrichous, according to their movement), which are hardly stainable with the Gram stain. No diplobacters were seen, neither were there any involution forms in cultures up to one month old.

S t r a i n 8138 behaved entirely different. After one or two days it grew into a very thick jelly-like mass which flowed down the slope of the slant. The cells are short, thick rods, no diplobacters.

None of these strains liquefied gelatine.

3. Aspect of the colonies on beer-gelatine.

After one week incubation at 20° C., strain 1 formed two types of colonies in Petri dishes with beer-12% gelatine. One was called "small", the other "large". Upon subsequent plating, each variety

yielded a mixture of both. After several platings apparently pure cultures of the "small" and the "large" variety were obtained. However, this was illusory, for when the cultures were plated out again several months later a mixture of the two types was obtained. This phenomenon was not encountered with the other strains.

After some experience the colonies of strain 1 "large", 838 and 8618 could easily be identified visually, since they resembled fried eggs, with a white centre. The colonies were circular, about 1—3 mm in diameter after one week growth, umbonate, with undulate edge, translucent, pale brown. The colonies of 1 "small" were circular, 1—2 mm in diameter, convex, with entire edge, smooth surface and translucent. The colonies of strain 3 were similar but white. The strain Davis resembled 1 "small", 8138 formed large viscous colonies, growing together over the entire surface of the plate.

4. Catalase content.

The presence of catalase was checked by adding one drop of a 3% H_2O_2 solution directly on the colony or on about 40 mg living bacteria from a centrifuge tube. The H_2O_2 solution was checked with a drop of blood or with a colony of a catalase-positive *Acetobacter*. A low-power binocular may advantageously be used. The strains 1 "large", 3, 838 and 8618 did not contain catalase since no gas bubbles were formed after 10—20 minutes. Strain Davis showed a weak but distinct positive reaction and there was an abundant oxygen formation with strain 8138.

5. Temperature optimum.

The bacteria were grown on beer-2% agar and incubated for one week at 20°, 25°, 30°, 35° and 40° C. The strains 1 "small", 1 "large", 3, 838 and 8618 grew best at 20—25°, rather well at 30°, hardly at 35° and not at all at 40° C.

Strain Davis grew very well at 20—30°, best at 35° and weakly at 40° C.

6. Growth on several substrates.

All the strains were grown on slants containing 1% Difco yeast extract, 2% agar, several carbon sources, and usually with $CaCO_3$. These media are identical to the ones described by FRATEUR (1950). The bacteria were also inoculated on the same medium

without an additional carbon source, as a control. The cultures were incubated at 30°C. and observed daily for two weeks. Aspects of growth and acid formation were recorded, as compared to the control, on which weak growth occurred but with no acid formation. Table 1 summarizes the results. There were no differences in the results between 1 "small" and 1 "large".

TABLE 1.

Substrate (concentration expressed as %)	Strains				
	1	3	838	8618	Davis
2 ethanol	good growth;	good growth;	good growth;	good growth;	good growth;
2 CaCO ₃	acid	acid	acid	acid	acid
0.1 Ca-acetate	good growth	—	—	—	—
2 Ca-acetate	—	—	—	—	good growth
0.1 Ca-lactate	good growth	—	—	—	good growth
2 Ca-lactate	good growth	good growth	good growth	excellent growth	good growth
10 L-arabinose 3 CaCO ₃	—	—	—	—	good growth; acid
2 glucose 2 CaCO ₃	—	—	—	—	growth; acid
10 glucose 3 CaCO ₃	—	—	—	—	good growth; acid
10 galactose 3 CaCO ₃	—	—	—	—	growth; acid
10 D-xylose 3 CaCO ₃	—	—	—	—	good growth; acid

These strains did not grow better than in the control, neither did they form acid on the following substrates: 10 D-arabinose, 3 CaCO₃; 2 rhamnose, 2 CaCO₃; 10 Ca-gluconate; 10 fructose, 3 CaCO₃; 10 fructose, 1 CaCO₃; 2 lactose, 2 CaCO₃; 2 maltose, 2 CaCO₃; 2 saccharose, 2 CaCO₃; 10 saccharose, 2 CaCO₃; 10 ribose,

3 CaCO_3 ; 10 raffinose, 2 CaCO_3 ; 2 starch, 2 CaCO_3 . The growth on 0.25% Difco yeast extract, 0.5 Difco peptone, 2% agar was extremely weak.

The strains 1,3, 838 and 8618 behaved in the same way, except for minor differences (on 0.1% Ca-acetate and 0.1 Ca-lactate). Strain Davis did not belong in the same group, as it was able to grow and form acid on glucose, galactose, L-arabinose and D-xylose. It also grew very well on 2% acetate. Strain 8138 produced acid from several carbohydrates.

The above results have to be compared with the list in FRATEUR's paper. This author reports growth on several carbohydrates, which suggest the presence of enzymic mechanisms for the decomposition of these substances. On the other hand, our table shows that the strains 1,3, 838 and 8618 are unable to grow on carbohydrates (strains Davis and 8138 are excluded from this discussion), at least, only to the extent of growth represented by the medium without carbon source, which is very slight. We believe that the lack of a control medium might be the explanation for the apparent discrepancy between the results, unless FRATEUR's strain was a completely different one.

7. The consumption of glucose and of gluconate.

FRATEUR reports that his strain of *A. peroxydans* displays the following characteristics on glucose: "levure-agar, 2% glucose, 2% CaCO_3 — 10 J. à 30° C.: strie blanc jaun, pas d'acide; levure-agar, 10% glucose, 3% CaCO_3 — 10 J. à 30° C.: bon développement, pas d'acide, irisation".

VISSER 'T HOOFT (1925) mentions that his strains produced crystals of CaCO_3 in a 1% yeast extract, 2% Ca-gluconate, agar medium. FRATEUR reports a similar observation. These positive results suggest that *A. peroxydans* may, under suitable conditions, develop the capacities to metabolize glucose and gluconate and that the glucose oxidation is effected by an enzyme system which contains at least one inducible member, since cells, grown on ethanol, are unable to oxidize this substrate (WIELAND and PISTOR, 1938; TANENBAUM, 1956). Yet, in view of the previously described discrepancies a thorough reinvestigation was required by the direct estimation of the substrate in the growth media.

The strains were grown in 1 liter flasks, containing 100 ml of a medium consisting of 1% Difco yeast extract, tap water and either

0.5, 2 or 10% glucose. The flasks were incubated at 30° C. on a shaking machine. After several days a marked turbidity indeed developed, which was however not superior to the one obtained in the same medium where glucose was absent. The glucose concentration was determined every second day over a period of two weeks by the method of Luff-Schoorl. Strains 1,3, 838 and 8618 did not consume glucose. Addition of phosphate, MgSO_4 , NaCl, trace elements and CaCO_3 did not influence the results. Strains Davis and 8138 formed much acid and after 2 days all glucose had disappeared. With strain Davis it could be shown that gluconic acid constituted the main endproduct, by several means: 1) by boiling with N HCl for 5 min. a lactone was formed, since it develops the typical brown colour with the hydroxylamine reagent (HESTRIN 1949); 2) this lactone behaved in the same way as the reference substance on paper chromatograms with the solvent n-butanol 3 / ethanol 1 / water 1; the position of the compound was revealed using the spray of ABDEL-AKHER and SMITH (1951); 3) the free acid, obtained after treatment with Amberlite IR 120 (H^+) behaved in the same way as the reference substance on paper chromatograms with the solvent methanol 6 / HCOOH 1 / water 3 using brom-cresolgreen as a spray reagent. *A. paradoxum* and *A. ascendens* were also unable to consume glucose, tested under the same conditions.

The strains were also inoculated in a medium containing 1% Difco yeast extract, 1.5% Ca gluconate and 0.02 mg % brom-cresole purple. Every second day the gluconate content was determined, after conversion into the lactone, with the hydroxylamine method of HESTRIN (1949). After one week there was only weak growth (not exceeding the one in the control medium without gluconate). After one week incubation, the gluconate had not decreased. On solid media prepared according to VISSER 't HOOFT, we also observed crystal formation, but they are crystals of Ca-gluconate, not of CaCO_3 . These are also formed on non-inoculated media. Prof. FRATEUR kindly informed us that in his experiments the crystals were really CaCO_3 probably formed from the yeast extract. Our results prove conclusively that our strains 1, 3, 838 and 8618 consumed neither glucose nor gluconate. The strains Davis and 8138 oxidized glucose with the formation of gluconate.

8. Overoxidation.

All the strains produce acetic acid in a medium, containing 1% Difco yeast extract, 2% ethanol. After one week to ten days all the acid is consumed again.

9. Acid-resistance.

It is a characteristic of real acetic acid bacteria that they are able to grow at a pH of 4.5 and lower. An Atkinson medium was prepared (see later), to which M/15 acetic acid and 2% ethanol were added. The medium was neutralized with NaOH, to pH 4.3, dispensed in 50 ml portions in 250 ml Erlenmeyers, sterilized, inoculated and incubated for at least one week at 30°C. All the strains grew very well.

10. Growth as hydrogen bacteria.

In view of the several unusual properties of *A. peroxydans*, in particular the oxidation of hydrogen with O₂, it seemed possible that this species might really be a *Hydrogenomonas*, with the additional property of oxidizing ethanol into acetic acid. Both liquid and solid media were used¹). A liquid medium with the same composition as used by SCHATZ and BOVELL (1952) for *Hydrogenomonas facilis*, was dispensed in 50 ml portions in small Erlenmeyers. This medium was heavily inoculated with young cultures of our strains from a beer-gelatine slant. The flasks were stored in a dessicator in a 10% CO₂, 30% air and 60% H₂ atmosphere and incubated for three weeks at 20°C. There was neither visible growth, nor an underpressure.

Silica-jelly plates were prepared with the same medium, also heavily inoculated with our strains and incubated in the same atmosphere in analogous conditions. One control plate was inoculated with river mud. Our strains did not grow, whereas the control showed distinct growth. Since none of our strains were able to grow autotrophically, they may not be considered as hydrogen bacteria. However, some hydrogen bacteria are fastidious and prone to lose autotrophy, and it would be interesting to repeat these experiments with freshly isolated cultures of *A. peroxydans*.

¹) We are indebted to Dr E. A. DAWES, Biochemical Dept., the University, Glasgow, for carrying out these experiments in our laboratory.

11. The oxidation of several substrates by resting cells.

The strains were grown in different media. We commonly used the medium described by ATKINSON (1956), containing per liter, 1.5 g NH_4Cl , 1 g Difco yeast extract, 5 g Na_2SO_4 , 4 g K_2HPO_4 , 200 mg $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 80 mg $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$, 80 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, and trace elements (0.35 mg $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$, 0.03 mg MoO_3 , 0.4 mg CuSO_4 , 9 mg ZnSO_4 , 0.4 mg $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$, 0.13 mg KI, 0.02 mg NH_4VO_3).

The solution was boiled and filtered while still hot, in order to avoid a turbid solution after sterilisation. This medium was supplied with 0.02 mg % bromcresol purple and pure D-lactic acid; it was neutralized with concentrated NH_4OH until a just visible purple colour. It was distributed in 200 ml portions in 1 l flasks inoculated with the bacteria from a 48 hours old slant. After one day incubation at 25 or 30° C., the pH became neutral, as shown by the colour of the indicator and was neutralized with either conc. HCl or lactic acid, until the pH dropped to 5.2 or below. Neutralisation was carried out twice a day. The bacteria were also grown on malt extract-2% ethanol, on 1% Difco yeast extract-1% glucose or on beer.

After two days the bacteria from several flasks were centrifuged, and washed twice with M/100 pH 7 phosphate buffer. The purity was checked by microscopic observation, Gram staining and the catalase test. The conventional apparatus for the Warburg experiments was used at a temperature of 30° C. Each vessel contained 50 mg living bacteria, 600 μmoles pH 7.0 phosphate buffer and 10 μmoles substrate making a total volume of 3 ml. The central well contained KOH. The respiration of the substrate was followed for 4-5 hours. At the end of the experiment, the pH of the suspension was roughly checked with an indicator.

11a. Results with strains 1, 3, 838 and 8618 (Table 2, Fig. 1).

The resting cells, after growth on media containing either ethanol, lactate or glucose were completely unable to oxidize glucose, gluconate, 2- and 5-ketogluconate, glycerol, sorbitol and fructose. We recall here that they were also unable to grow on some of these substances, as well as on other carbohydrates.

Ethanol was oxidized by ethanol- or lactate- grown cells with the uptake of 2.3—2.6 mole O_2 per mole substrate (1.3 mole O_2 with strain 3). For complete oxidation 3 mole O_2 was required,

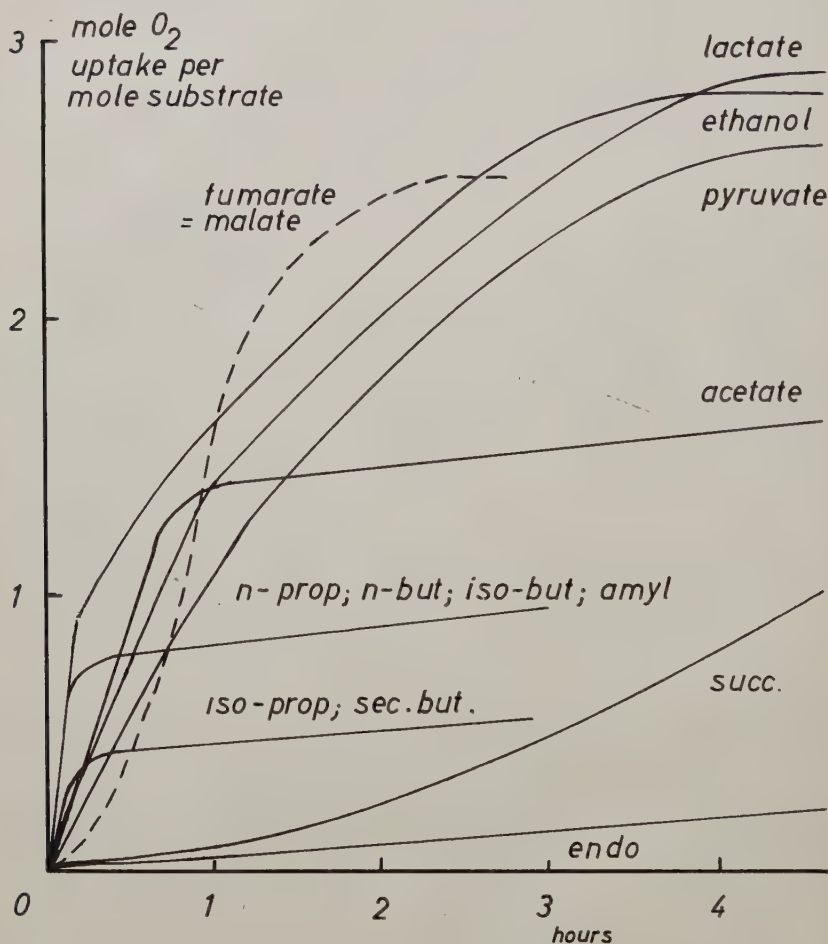


Fig. 1. The oxidation curves of several substrates by resting cells of *A. peroxydans* 8618, grown on Atkinson-1% ammonium lactate medium for 2 days at 30° C. Content of the Warburg vessels as described in the text. Abbreviations: succ. (succinate); n-prop, n-but, iso-but, amyl, iso-prop, sec. but (the corresponding alcohols).

showing that 13–23% of the ethanol was assimilated. Since these cells oxidized this substrate also when they had been grown on DL-lactate or on yeast extract alone, ethanol oxidation must be considered a constitutive property. Very often the respiration curve showed an inflection after the uptake of 1 mole O₂: acetate was thus formed at a rapid rate and oxidized only after nearly all the ethanol was gone. Cells grown on 1% yeast extract oxidized

TABLE 2.

Oxygen uptake with resting cells of the bacteria under investigation in the presence of several substrates. Content of the Warburg vessels as mentioned in the text. The results are expressed as mole O_2 consumed per mole substrate and corrected for the endogenous O_2 .

Substrates	Strains				
	1	838	8618	3	Davis
Na-DL-lactate	1.9-2.3	1.9	2.7	1.3	1.4-1.7
ethanol	2.3-2.6	2.1-2.4	2.6	1.1-1.3	0.9-1.4
Na-pyruvate	2.2	2.1	2.4	2.3	1.6
Na-acetate	1.3	1.3	1.4	1.3	>0.2
Na-succinate	>0.3	>0.3	>0.7	>0.1	2.4
Na-L-malate	2.6	2.6	2.6	>1.5	>2.2
Na-fumarate			2.5	0	
glucose	0	0	0	0	0.5
methanol	0.1	0.2	0.2	>0.4	
<i>n</i> -propanol	0.8	0.9	0.9	0.85	
<i>n</i> -butanol	0.9	0.9	0.8	0.9	
<i>iso</i> -butanol	0.8	0.8	0.7	0.8	
<i>n</i> -amylalcohol	0.9	0.9	0.9	0.85	
<i>iso</i> -propanol	0.5	0.4	0.4	0.5	
<i>sec.</i> butanol	0.5	0.5	0.45	0.5	
<i>tert.</i> butanol	0	0	0	0	

ethanol to the same extent when one day old; two days old cells scarcely beyond acetate, since 0.9-1.2 mole O_2 was taken up.

Acetate oxidation proceeded with the uptake of 1.3 mole O_2 per mole substrate. The extent of acetate assimilation would therefore be about 35%.

DL-lactate was oxidized with the uptake of 1.9-2.7 mole O_2 (1.3 mole with strain 3). Cells grown on lactate or on ethanol oxidized DL-lactate to the same extent and at approximately the same rate. However, cells grown on yeast extract alone oxidized lactate only with the uptake of 0.2 mole O_2 . Pyruvate oxidation proceeded nearly to completion, with 2.2-2.4 mole O_2 . Succinate was oxidized slowly (only 0.7 mole O_2 after 5 hours). L-malate was oxidized better, up to 2.6 mole O_2 . Fumarate was very well oxidized by strains 1, 838 and 8618, but not by strain 3. Citrate was not oxidized.

The varieties "small" and "large" of strain 1 behaved in exactly the same fashion.

These strains readily oxidized *n*-propanol, *n*-butanol, *iso*-butanol

and *n*-amylalcohol with the uptake of nearly 1 mole O_2 , corresponding to the propionic, *n*-butyric, *iso*-butyric and *n*-valeric acids. *Iso*-propanol and *sec.* butanol were oxidized to the corresponding acetone and ethyl-methylketone stage. Methanol was oxidized very slowly.

All our strains oxidized H_2 in the Warburg, in a gas-atmosphere containing 80% H_2 and 20% air. In the collection of bacteria there were unfortunately no strains, which were unable to oxidize H_2 , which could be used for comparison purposes.

11b. Results with strain Davis.

These cells behaved in a different way. When grown on either Atkinson-2% ethanol, Atkinson-1% ammonium lactate, Atkinson-1% glucose or 1% yeast extract-1% glucose, glucose was always oxidized with the uptake of 0.5 mole O_2 . The medium became acid and gluconic acid could be demonstrated by paper chromatography. These cells were unable to oxidize gluconate, 2- and 5-ketogluconate, fructose or sorbitol. In contrast to the previous strains, the oxidation of glycerol, took up approximately 2.7 mole O_2 , thus proceeding far beyond the dihydroxyacetone stage. The results with the other substrates are summarized in Table 2.

12. The oxidation of some phosphoric esters by cell-free extracts.

The complete inability of the bacteria of strain 1, 3, 838 and 8618 to grow on, to consume or to oxidize several hexoses, pentoses and derivatives, suggests that they might lack the enzyme systems of either the glycolysis, the hexose monophosphate oxidative cycle (shunt) or a related mechanism. On the other hand, part of the cell wall undoubtedly contains some sugars, and ribose phosphate is almost certainly involved in the RNA formation. This requires some system for the synthesis of hexose and pentose phosphoric esters from ethanol or lactate. The hexose monophosphate oxidative cycle seems the most likely to accomplish this task. To test for the presence of this mechanism, we have investigated the oxidation of glucose-6-phosphate, gluconate-6-phosphate and ribose-5-phosphate by crude cell-free extracts of strains 3 and 8618.

The bacteria were cultured as usual on the Atkinson-lactate medium, centrifuged, washed, suspended in M/100 pH 6.0 phosphate buffer (1 gram wet cells in 15 ml) and Raytheonized for 15 min.

The suspension contained only few intact cells after this treatment, which was followed every five minutes by a methylene blue or Gram staining. The suspension was centrifuged at 4° C. at top speed in the Servall Angle Head centrifuge for 1—2 hours. The clear, yellowish supernatant was used as crude cell-free extract.

12a. TPN- and DPN-linked dehydrogenases.

The Beckman spectrophotometer, model DU, was used at a wave-length of 340 $m\mu$. With strain 8618 there was a distinct G6P-dehydrogenase activity when TPN and DPN together were used. No effort was done to determine the exact coenzyme requirement. 6PG or R5P were only slightly oxidized under these conditions (Fig. 2).

In strain 3 there was a TPN-linked G6P and R5P oxidation, but there was no reaction with 6PG (Fig. 3). G6P and R5P were not oxidized with DPN. There was a weak reaction with 6PG and DPN.

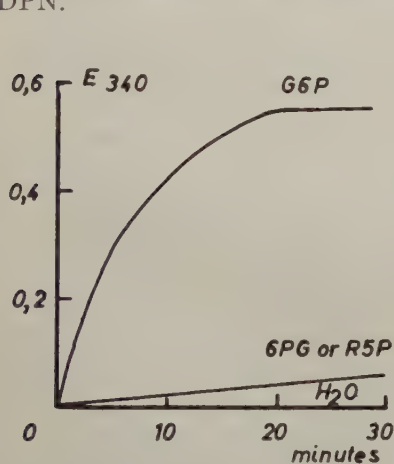


Fig. 2. The oxidation of G6P, 6PG and R5P by coenzyme-linked enzymes with cell-free crude extract of strain 8618. Each Beckman cuvette contained 0.1 ml crude extract (corresponding to about 30 mg living bacteria), 60 μ moles Mg^{++} , 0.3 μ mole TPN and 0.3 μ mole DPN in 2.9 ml M/50 pH 8.0 Tris-HCl buffer. At zero time 2 μ moles substrate in 0.1 ml were added.

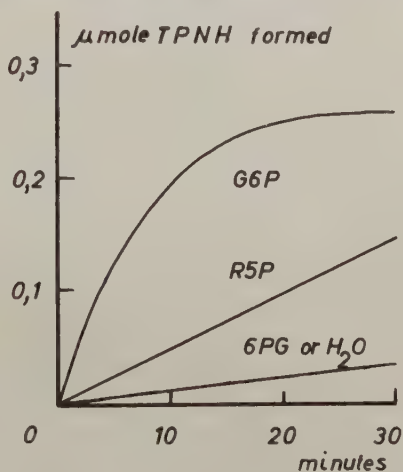


Fig. 3. The oxidation of G6P, 6PG and R5P by TPN-linked enzymes with crude cell-free extracts of strain 3. Each Beckman cuvette contained: 60 μ moles Mg^{++} , 0.5 ml extract (corresponding to about 160 mg living bacteria), about 0.3 μ mole TPN in 2.9 ml M/45 pH 7.4 Tris-HCl buffer. At zero time 2 μ moles of the substrate in 0.1 ml were added.

12b. Oxidations with phenazine in the Warburg.

In the presence of N-methyl-phenazinium-methylsulfate and TPN or DPN, the extracts of both strains were able to oxidize the three key intermediates of the hexose monophosphate oxidative cycle. The oxidations proceeded too slowly to determine the final equations. With strain 8618 at least 1.1 mole O_2 with G6P, 0.6 mole O_2 with 6PG and 0.4 mole O_2 with R5P were taken up per mole substrate (Fig. 4).

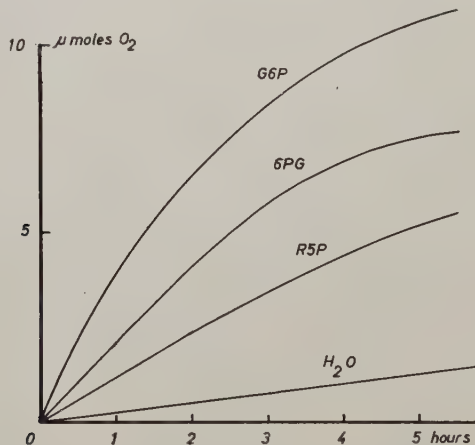


Fig. 4. The oxidation of G6P, 6PG and R5P by cell-free crude extracts of strain 8618. Each Warburg vessel contained M/45 pH 7.5 Tris-HCl buffer, 60 μ moles Mg^{++} , 0.6 μ mole TPN, 0.6 μ mole DPN, 2.7 μ moles N-methyl-phenaziniummethylsulphate, 10 μ moles substrate, 1 ml crude extract (corresponding to 50 mg living cells). Final volume 3 ml. KOH in the central well. Temperature 30° C.

With strain 3 the reactions were studied into more detail. None of the phosphoric esters were oxidized with the phenazine in the absence of TPN and DPN. G6P was oxidized in the presence of TPN with the uptake of 0.56 mole O_2 per mole substrate, indicating the formation of 6PG. The oxidation in the presence of DPN proceeded at approximately 1/10 of the rate with TPN. 6PG was oxidized slowly with DPN, not with TPN. After 5 hours 0.39 mole O_2 per mole 6PG had been consumed and the reaction still continued. R5P was oxidized slowly with TPN and perhaps very weakly with DPN. After 5 hours with TPN 0.3 mole O_2 per mole R5P had been used and the oxidation still continued. When the crude preparation was centrifuged for 2 hours at 100,000 g and 4° C. in

the Spinco-centrifuge, the particles were removed. All the enzyme activity was retained in the supernatant.

DISCUSSION.

The strains 1, 838 and 8618 behave identically in nearly every respect. Strain 3 is very similar and differs only in a few details. They are all short rods, mostly diplobacters, strictly aerobic, motile, Gram negative, catalase negative and do not form involution forms. Their growth and physiological characteristics on different culture media are nearly indistinguishable. Strain 1 is unstable and forms two varieties called "small" and "large". In contrast with the others, strain 3 grows as white slants and forms white colonies. The temperature optimum is about 20—25° C. They are all acid-resistant, they grow only to an appreciable extent on lactate and ethanol in a synthetic medium, on 1% yeast extract or on beer. They are unable to grow on any carbohydrate or derivative. It is remarkable that they cannot grow on glucose, consume it or oxidize it. They belong thus to the very rare bacteria which are unable to metabolize this substrate, which is such a common carbon source for most other bacteria. This inability is the more remarkable since the other *Acetobacter* species (with the exception of *A. paradoxum* and *A. ascendens*) consume and oxidize glucose and other sugars rapidly. These bacteria are unable to grow in the conditions for hydrogen bacteria. The resting cells oxidize readily DL-lactate, pyruvate, ethanol, acetate, some members of the Krebs cycle and several alcohols, the latter to the corresponding acid or ketone stage. There are some minor differences in the oxidative properties between strain 3 and the three others.

All these results suggest that *A. peroxydans* contains a core of constitutive enzymes for the oxidation of C₃ and C₂ compounds with the formation of CO₂ and water, *viz.*, from lactate, pyruvate and ethanol over acetaldehyde, acetate and the Krebs cycle or a modification therefrom. Indeed, ATKINSON (1956a) and TANENBAUM (1956) have studied some enzymic aspect of ethanol and pyruvate metabolism and the possible role of the Krebs cycle.

A. peroxydans is able to oxidize G6P, 6PG and R5P at an appreciable rate with TPN and DPN linked enzymes. This occurs most likely through the hexosemonophosphate oxidative cycle. Since these strains are unable to metabolize any sugar, this means

that they are genetically unable to form permeases or kinase for these carbohydrates. The H.M.P. cycle would therefore have only an anabolic function for the synthesis of hexoses and pentoses, required for cell wall, RNA formation, etc.

We regard the strains 1, 3, 838 and 8618 as the real *A. peroxydans*. The taxonomic position of this species was not clear up to now (see e.g. Bergey, Sixth Edition; it was removed from Bergey's Seventh Edition). From the above results it follows that this species has been classified correctly in the genus *Acetobacter*. The comparative biochemistry and enzymology of the peroxydans group, in relation to the members of the other groups will throw further light on this opinion. One can imagine that *A. peroxydans* originated in the course of evolution from mesoxydans or oxydans bacteria by mutations, involving the loss of catalase and kinases or permeases, such that polysaccharides, C₆ and C₅ sugars and their derivatives could not be introduced into the intermediary metabolism. One point in favour of this hypothesis is an observation of FRATEUR (1952), who isolated catalase negative strains of *Acetobacter* having otherwise all the biochemical characteristics of *A. rancens* and *A. mesoxydans*.

The strains Davis and 8138 can not be regarded as *Acetobacter peroxydans*. They are morphologically different (short rods, no diplobacters). They contain catalase, have a different temperature optimum, grow on several sugars and oxidize e.g. glucose with the formation of gluconic acid. The strain Davis very likely contains only the glucose-oxidizing enzyme and grows really at the expense of the yeast extract. This opinion is stressed by the Warburg experiments with cells, grown on yeast extract-glucose, which oxidize also glucose quantitatively into gluconate and not beyond that stage. They are also unable to oxidize acetate to an appreciable extent, although some Krebs cycle intermediates are oxidized. The real taxonomic position of these strains has not been further investigated.

S u m m a r y.

Some physiological and biochemical properties of several strains of *Acetobacter peroxydans* have been studied. Their morphology, the aspects of growth on beer-gelatine slants and the colony type are described. The cells are catalase negative and acid resistant.

The temperature optimum is 20—25°C. They grow readily on ethanol and on lactate, or on yeast extract alone, but not on carbohydrates and derivatives. They do not consume or oxidize glucose or gluconate. They are overoxidizers. None of these strains was able to grow as hydrogen bacteria.

Resting cells oxidize lactate, pyruvate, ethanol, acetate, some Krebs cycle intermediates and several alcohols. Cell-free extracts oxidize glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate in suitable conditions. These results favour the taxonomic position of this species as a member of the genus *Acetobacter*. The results are discussed.

Acknowledgement.

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(Koninklijke/Shell-Laboratorium, Amsterdam).

n-ALKANE OXIDATION BY A *PSEUDOMONAS* STUDIES ON THE INTERMEDIATE METABOLISM

by

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INTRODUCTION.

Since normal alkanes are rather refractory towards most chemical agents, — at least at room temperature — the attack on these compounds by micro-organisms represents an interesting biochemical phenomenon. Among the problems posed by the initial steps of microbiological alkane oxidation, the point of primary attack on the molecule is an intriguing one. Little work has, however, been published on this special subject.

TRECCANI *et al.* (1955) concluded from manometric experiments with *Ps. aeruginosa* that alkane oxidation proceeds by steps which — apart from the initial ones — are identical with those occurring in the breakdown of the corresponding fatty acids. Though the point of initial attack is not especially mentioned, the Italian group regards the oxidation of a terminal methyl group as the most probable pathway (ARNAUDI, 1955).

Identification of heptylic and succinic acids among the fatty acids isolated from a culture of *Ps. aeruginosa* growing on heptane was interpreted by SENEZ (1956) as indicating that primary oxidation of a terminal carbon could be accompanied by the oxidation of both end groups.

Data obtained by KALLIO *et al.* (1954/1956) — who were guided by multiple adaptation experiments — appeared to be unrelated, though a pattern of one-end peroxidation emerged. An intermediate was isolated from a culture of *Nocardia* oxidizing hexadecane. Mass spectrophotometry indicated that the isolated intermediate was cetyl palmitate. The ester is believed to be formed from two molecules of hexadecanal by a biological Tishchenko-type reaction (KALLIO, 1957).

LADD (1956) found the ready oxidation of undecanon-2 by a *Corynebacterium* to contrast with the poorer and inconsistent oxidation of *n*-decyl aldehyde. This suggests that oxidation of *n*-decane might proceed at a position other than the terminal carbon. On the other hand terminal oxidation was perhaps indicated by co-oxidation experiments in the respirometer of *n*-decane with *n*-decyl alcohol, *n*-decyl aldehyde and *n*-decylic acid respectively. In no case did the co-oxidation rate equal the sum of individual rates.

Recent investigations of HARRIS (1957) exclude primary alcohols from the metabolic pathway of alkane breakdown by a *Micrococcus*. Secondary alcohols and ketones tested are also characterized by low oxidation rates, whereas monocarboxylic fatty acids are readily oxidized.

HARRIS — after considering spatial configuration models — tentatively suggested that exposed terminal methyl groups at least facilitate alkane oxidation.

The oxidation of long-chain alkylbenzenes by *Nocardia* was investigated by WEBLEY *et al.* (1956). The resulting phenyl-substituted fatty acids (which were isolated) could be explained by an initial attack at any of the ($\omega-2n$) C atoms of the chain ($n = 0, 1, 2$, etc.). To WEBLEY and his group terminal oxidation ($n = 0$), seemed the most probable route.

Summarizing, it appears that the latter route is generally considered to be the most likely, though no definite proof has been given.

No intermediates of lower oxidation level than fatty acids were identified in *n*-alkane oxidation.

Experiments on the intermediate metabolic pathway of *n*-alkane respiration by a *Pseudomonas* have been undertaken by the present authors. Emphasis has been laid on the initial steps and the point of primary attack on the molecule. The first results of these studies are presented below.

MATERIALS AND METHODS.

Organism.

A bacterium was isolated aerobically from Y-harbour (Amsterdam) water by enrichment in a liquid mineral medium¹) and kerosene

¹) KH_2PO_4 4.5 g; Na_2HPO_4 4.8 g; KNO_3 1.0 g; FeSO_4 5 mg; H_2O 1000 ml. pH=6.9.

as sole source of carbon. Purification of the microorganism was carried out by passing it through the same kerosene medium and finally by plating it on Difco nutrient-agar and isolating a single colony. Stock cultures were maintained on nutrient-agar slants; working cultures were kept on mineral-agar and in alkane vapour.

The motile (by polar flagella), Gram-negative, gelatin-liquifying straight rods produced pigments of greenish hue. It formed nitrogen as chief product from nitrate anaerobically. Ready growth was observed from 20°C. up to 37°C. The microorganism is believed to be closely related to — if not identical with — *Ps. aeruginosa*.

The production of cells for Warburg experiments was accomplished by the inoculation of ca. 6 Petri dishes containing mineral-agar. In the lid of the dishes a strip of filter paper provided with some drops of hexane or heptane was placed. Incubation for 16 hrs at 30°C. was performed in closed tins. After incubation the cells were harvested, washed by centrifugation and resuspended in phosphate buffer. The density of the suspension was standardized by the use of a spectrophotometer (600 m μ).

C h e m i c a l s.

Hydrocarbons used were model compounds purchased from Phillips or A.P.I. Alcohols, aldehydes and acids were obtained from various sources in the highest purity available and redistilled if necessary.

M e t h o d s.

Warburg techniques were used throughout this study, as described by UMBREIT (1951). Warburg's direct method (UMBREIT, 1951) was followed for the determination of the respiratory quotient. Simultaneous adaptation was studied by the determination of oxygen consumption rates of suspected intermediates as described by STANIER (1950).

EXPERIMENTAL.

I. Determination of the Respiratory Quotient.

R.Q. determinations were carried out in Warburg respirometers with heptane as a carbon source for resting *Pseudomonas* cells.

In series I the bacterium was suspended in water. In series II and III a phosphate buffer of pH 6.0—6.1 has been used. Though

undertaken under identical conditions, the duration of the experiment was extended from ca. 2 hrs up to ca. 4 hrs in series III. In the latter series corrections were made for the building-up of additional heptane pressure as a result of a slow diffusion of heptane vapour into the capillary tubes of the manometer. This process takes a few hours and has, consequently, not ceased after 20 minutes of temperature equilibration. The results of the series are given in Table 1.

TABLE 1.

R.Q. of resting *Pseudomonas* cells during respiration of *n*-heptane.

Series							Mean
I	0.61	0.61	0.65	0.61	0.61	—	0.62
II	0.64	0.68	0.65	0.55	0.66	—	0.63
III	0.59	0.60	0.67	0.66	0.56	0.64	0.62

Experimental details: See under materials and methods. Warburg flask main compartment: 1 ml phosphate buffer pH 6.0-6.1, 0.1 ml heptane. Centre well: 0.2 ml 10% KOH or nil. Side arm: 1 ml bacterial suspension (1.3 mg cells dry wt.). Total volume: 2.3 ml. Temperature: 30°C.

Though some spread in the results was encountered, the mean value of 0.62 agrees fairly well with a theoretical value of 0.64 as calculated for complete heptane oxidation.

It was observed that during the experiment the R.Q. invariably increased, starting sometimes as low as 0.54 to settle at the figures given in the table. The R.Q. is calculated from the results obtained during the last 90 minutes.

The only figure found in the literature for heptane was 0.47, as reported by JOHNSON *et al.* (1942) for *Bacterium aliphaticum*. Most studies have been performed with higher alkanes and have resulted in figures approximating to the theoretical value (LADD 1956, HARRIS 1957).

Our experiments do not indicate that heptane oxidation is accompanied by an appreciable formation of products, except carbon dioxide and water. They agree with what is generally assumed for *n*-alkanes; *viz.*, complete oxidation.

II. Multiple Adaptation Studies.

A. Oxidation rates of oxygenated hexane derivatives.

Washed *Pseudomonas* cells adapted to hexane were investigated

as regards their capacity for oxidizing various C_6 compounds. Compounds showing an immediate uptake of O_2 at a rate comparable to hexane are regarded as possible intermediates in the process. To summarize results, hexane was run in each experiment and its oxidation rate was arbitrarily taken as 100. Thus oxidation rates of the substrates tested are expressed as a percentage of hexane oxidation. Inconsistent results are indicated by quotation of the range observed, instead of a mean value.

It can be seen from table 2 that hexanol-1, hexanal and caproic acid could possibly function as intermediates. Hexanediol-1,6 is not regarded as such because of the low oxidation rate of adipic acid.

Hexenes are slowly oxidized, which indicates that the oxidation process is not initiated by a dehydrogenation process. One could imagine that hexenes ought to be presented as some activated compound but alcohols expected to arise from hexene oxidation also show low oxidation rates. Hexanediols, though never to be regarded as primary intermediates, were tested because BRUYN (1954) reported diol formation from alkenes by a yeast.

TABLE 2.

Oxidation of C_6 compounds by hexane-adapted *Pseudomonas* cells (Figures presented as percentage of hexane oxidation rate).

Hexanol-1	> 100	Hexanal	> 100	Caproic acid	> 100
Hexanol-2	30	Hexanon-2	15-35		
Hexanol-3	45				
Hexanediol-1,2	65				
Hexanediol-2,3	25				
Hexanediol-1,6	100			Adipic acid	10
Hexanediol-2,5	15				
Hexene-1	40	Hexene-3 (cis)	25	Hexane	100
Hexene-2 (cis)	25	Hexene-3 (trans)	25	Blank	15
Hexene-2 (trans)	20				

Substrate concentrations: Hexane and hexenes 0.1 ml per flask, other compounds 13μ mole per flask. See also under Fig. 1.

The conclusion from table 2 is that hexane might be oxidized by the route of hexanol-1, hexanal and caproic acid. This is stressed by the observation that hexanol-1 is not oxidized at an appreciable rate by glucose-peptone or caproic-acid-grown cells.

B. Fatty acid degradation by hexane- and heptane-adapted cells.

Selectivity of hexane-adapted cells towards the fatty acids with odd and even numbers of C atoms was taken by TRECCANI (1955) (*Ps. aeruginosa*) to indicate that β -oxidation is the principle pathway in fatty acid degradation. Selectivity can only be explained if the initial point of attack on the alkane molecule is at a specified C atom. Thus, low oxidation rates of odd-numbered acids indicate a primary attack on C_1 or C_3 .

The experiments of TRECCANI were repeated with heptane- and hexane-grown cells. Oxidation rates of C_3 — C_6 alcohols and aldehydes were also measured, as it was understood that free acids are not intermediates in fatty acid metabolism. Results obtained with the acid series (Fig. 1) compared favourably with results obtained by TRECCANI. Alternating oxidizability by hexane-grown cells is clearly demonstrated.

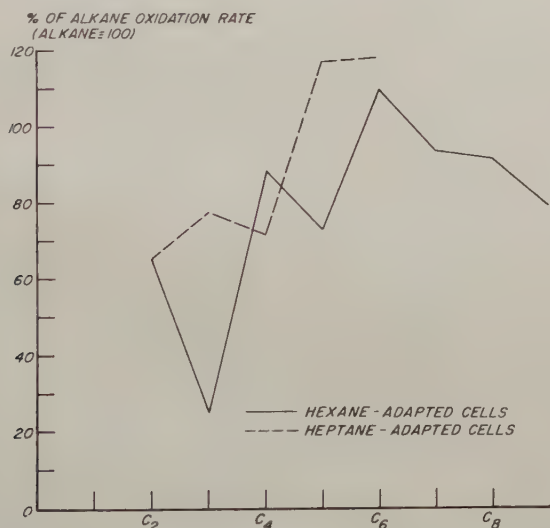


Fig. 1. Oxidation rates of homologous series of fatty acids (C_2 — C_9) by heptane- and hexane-grown *Pseudomonas* cells. Results expressed as percentage of heptane or hexane oxidation rate respectively.

Experimental details: Warburg flask main compartment: 1 ml Sørensen phosphate buffer pH 6.8 Substrate: 0.1 ml alkane or 0.1 ml fatty acid Na salt solution. Side arm: 1 ml bacterial suspension in buffer approximating 1.3 mg bacteria (dry wt). Centre well: 0.2 ml 10% KOH. Total volume 2.1 ml + 0.2 ml. Temp. 30°C. Final substrate conc. 27 μ mole per flask.

It was also found that, for instance, heptylic acid oxidation is appreciable as compared with that of propionic acid. This phenomenon was taken by TRECCANI to indicate increasing participation of some C_1 breakdown system (e.g. decarboxylation) — besides that of β -oxidation — towards the higher acids. This postulation, however, is not a necessary one if either:

- propionic acid accumulates to some extent in the medium during heptylic acid oxidation by hexane-grown cells, or
- if *Pseudomonas* cells are equipped with a constitutive enzyme system for the metabolism of propionyl-CoA (e.g. carbon-dioxide fixation). (FLAVIN 1957a, MARTIN 1957).

Results obtained in the alcohol and aldehyde series as represented in Fig. 2 show that β -oxidation persists in the oxidation of these compounds (see also Fig. 3 and 4).

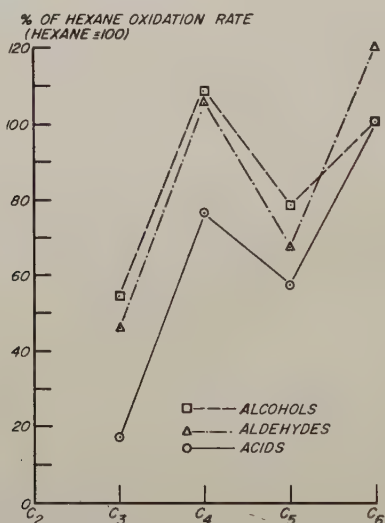


Fig. 2. Oxidation rates of C_3 – C_6 primary alcohols, aldehydes and acids by hexane-grown *Pseudomonas* cells. Results expressed as percentage of hexane oxidation rate.

Experimental details: Substrate conc.: $27 \mu\text{mole}$ per flask or 0.1 ml hexane. See also details under Fig. 1.

In addition it can be seen that propanol-1 oxidation by hexane-grown cells is appreciable as compared with that of propionic acid.

Propanol oxidation seems to be complete (O_2 consumption per $5.33 \mu\text{mol}$ calculated $538 \mu\text{l}$, found $482 \mu\text{l}$ or 90%). Thus no pro-

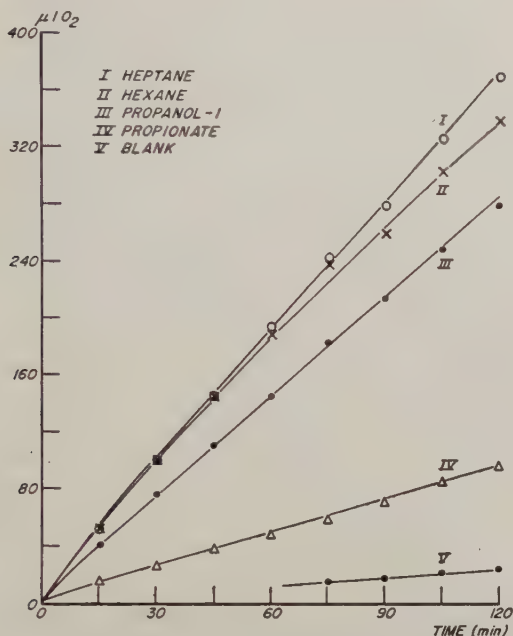


Fig. 3. Propanol and propionate oxidation by hexane-grown cells.
Experimental details: Substrate conc.: 54 μ mole per flask. See also details under Fig. 1.

pionic acid accumulation has to be assumed in heptylic acid oxidation where the number of heptylic acid molecules oxidized will be less than the number of propanol molecules, conditions being identical. Consequently, in heptylic acid oxidation¹⁾ the propionyl-CoA oxidising system will be loaded less intensively, because other factors such as the respiratory chain capacity are becoming limiting. In agreement with the above, heptylic acid oxidation also seems to be complete (O_2 consumption/5.0 μ mol calculated 1063 μ l, found 828 μ l or 78%).

No indications being present for propionic acid accumulation to any appreciable extent, the supposition under b) was further investigated with respect to the constitutive character of the propanol oxidizing system found. Fig. 5 shows that propanol-1 is oxidized at an appreciable rate by ethanol-grown cells. Again oxidation was complete (O_2 consumption/10.68 μ mol: calculated 1077 μ l, found 1100 μ l or 100.2%). A constitutive character of the enzymes responsible for propanol oxidation is thus indicated.

¹⁾ Or in general towards longer chain length.

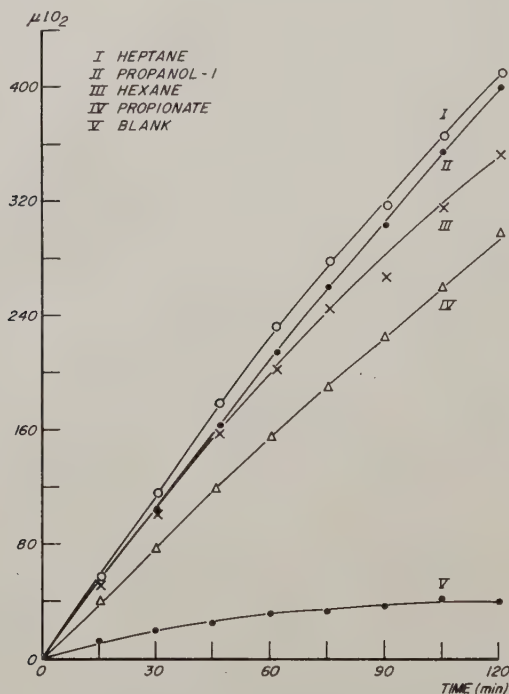


Fig. 4. Propanol and propionate oxidation by heptane-grown cells. Experimental details: Substrate conc.: $54 \mu\text{mole}$ per flask. See also details under Fig. 1.

It remains to be explained why propionic acid oxidation was found to be extremely slow as compared with propanol oxidation. It is tentatively suggested that the initial steps in propionic acid oxidation (first step Co A-compound formation and second step carbon dioxide fixation) are dependent on ATP free energy of hydrolysis (FLAVIN 1957*b*). The first step is bypassed when propanol is the substrate, the second step is postponed when valeric acid, and even more so when heptylic acid is metabolized.

We believe that extremely low oxidation rates of propionic acid, (and to some extent valeric acid), as compared with the corresponding alcohols, is attributable to such factors as quoted above and that alternating oxidizability of short-chain compounds ($\text{C}_3\text{—C}_6$) is found to be exaggerated when measured with free acids.

It can be concluded from the experiments described above on fatty acid degradation that attack on the alkane molecule occurs

at a specified C atom *viz.*, C₁ or C₃. Attack on C₂ is excluded also by low oxidation rates of hexanol-2 by hexane-grown cells. Attack on C₃ is highly improbable because of a low oxidation rate of hexanol-3.

The above suggests that the experimental facts may be satisfactorily explained on the basis of a terminal attack, followed by a β -oxidative degradation and that there are no reasons for assuming the operation of a C₁ breakdown mechanism.

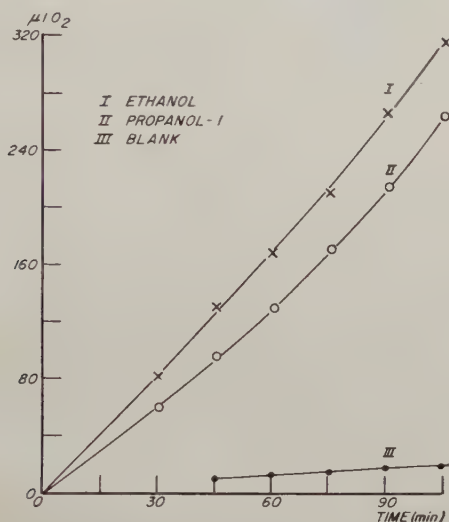


Fig. 5. Propanol oxidation by ethanol-grown cells.

Experimental details: Substrate conc.: 54 μ mole per flask. See also details under Fig. 1.

Summary.

1. A *Pseudomonas* oxidizing *n*-alkanes adaptively was used in experiments on hexane and heptane degradation.
2. The R.Q. of heptane respiration by resting heptane-adapted cells was found to be 0.62. No appreciable accumulation of products except CO₂ and H₂O seems to occur.
3. Alkane oxidation proceeds by oxidation of one terminal methyl group, leading to the corresponding alcohols, aldehydes and fatty acids as indicated by multiple adaption studies.
4. Oxidation of intermediate fatty acids by a β -oxidative mechanism is clearly indicated.

5. A constitutive propionyl-group oxidizing system was found to be present, though underdeveloped in hexane-grown cells.
6. No indications have been encountered so far to suggest the operation of a second (C_1) breakdown principle in the metabolism of alkanes to CO_2 and H_2O .

A c k n o w l e d g e m e n t s.

We are greatly indebted to the late Prof. A. J. KLUYVER (Delft) for his keen interest and valuable advice during the first part of these studies.

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(Laboratoire annexé au service de Dermato-Syphiligraphie des Hôpitaux Universitaires de Bruxelles et Service d'Hygiène de la Faculté de Médecine de Bruxelles).

L'ACTIVITE LIPOLYTIQUE MICROBIENNE DECELEE PAR LA METHODE DE SIERRA AVEC REFERENCE SPECIALE AU *M. PYOGENES* VAR. *AUREUS*

par

A. DELMOTTE

(Reçu le 24 Avril 1958).

1. INTRODUCTION.

Le principe de la précipitation en cristaux du sel de calcium d'un acide gras sous l'influence d'une enzyme a été appliqué d'une manière simple par SIERRA (1957) à la mise en évidence de l'activité lipolytique des microorganismes. Pour ce faire, du chlorure de calcium et un détersif non-ionique de la série des Tweens sont ajoutés au milieu de base. Les Tweens contiennent dans leur molécule un acide gras en liaison ester-acide. Si la souche microbienne possède une enzyme lipolytique, la précipitation des cristaux de savon calcique sera visible sous forme d'un halo opaque autour des colonies, ou même, si l'activité lipasique est intense, sous forme de cristaux visibles à l'oeil nu. Ces derniers peuvent être retrouvés, dans tous les cas, au microscope.

Les Tweens sont des dérivés des Spans. Ceux-ci, esters gras des hexitanes ou hexides formés par déshydratation du sorbitol (COLSON, 1954) se prêtent mal à l'expérimentation bactériologique, car ils résistent mal à la stérilisation, sont insolubles ou peu solubles dans l'eau et troublent les milieux gélifiés. Au contraire, les Tweens, hydrosolubles, résistent à la stérilisation, et formant des solutions claires, conviennent parfaitement. Ils sont obtenus par condensation de l'oxyde d'éthylène sur le radical -OH libre des Spans (COLSON, 1954). A cause de leur caractère hydrosoluble, ils permettent un contact intime entre l'acide gras et les bactéries (SIERRA, 1957).

SIERRA a utilisé le Tween 20 ou monolaurate de sorbitane polyéthoxylé, le Tween 40 ou monopalmitate de sorbitane polyéthoxylé, le Tween 60 ou monostéarate de sorbitane polyéthoxylé, le Tween 80 ou monooléate de sorbitane polyéthoxylé. A cette série, l'Emullat R40 ou ester polyéthylèneglycol d'un glycéride de l'acide ricinoléique et l'Emullat 06 ou oléate d'hexaéthylèneglycol, ont été ajoutés.

Il est cependant évident que, lorsque la précipitation du sel calcique a lieu, il n'est pas absolument sûr qu'il s'agisse d'une lipase car de nombreux sels de calcium d'acides organiques sont insolubles. La preuve ne pourrait être donnée que par l'isolement du savon calcique à partir de la préparation. Toutefois, un argument de plus en faveur de l'existence d'une lipase consiste dans le fait qu'il est possible d'observer les mêmes halos d'opacification et les mêmes cristaux lorsqu'on substitue aux Tweens 20, 40 ou 80, les Spans 20, 40 ou 80. Les acides gras des Tweens et des Spans de même indice numérique sont identiques. Le Span 60 est malheureusement très instable en solution aqueuse et ne se prête pas à l'expérience. Pour rappel, une "lipase" suivant NACHLAS et SELIGMAN (1949) hydrolyse des esters d'acides gras à longue chaîne, y compris les graisses et les huiles.

2. ACTIVITÉ DE LA LIPASE DE GERME DE BLÉ SUR LES DÉTERSIFS.

a) Réactifs.

1. lipase de germe de blé (préparation A. P. 5519 de Worthington Chemical Corporation) diluée à 10% en eau physiologique stérile;

2. bouillon ordinaire;

3. solution stérile de chlorure de calcium à 1%₀₀ en eau physiologique;

4. solution stérile à 10% en eau distillée des détersifs Tween 20, Tween 40, Tween 60, Tween 80, Emullat R 40.

b) Technique.

Dans une première série témoin, 1cc d'une solution contenant 1/10.000 de chlorure de calcium et 1% du détersif envisagé est versée stérilement dans 5 tubes à hémolyse. Trois autres tubes témoins sont constitués respectivement par un mélange bouillon stérile+lipase à 1%+chlorure de calcium, bouillon stérile+lipase à 1% et bouillon stérile+chlorure de calcium.

Dans la série expérimentale, chaque tube contient une solution

stérile de bouillon dans laquelle se trouvent 1/10.000 de chlorure de calcium, 1 % de lipase et 1 % du détersif envisagé.

c) Résultats.

Après séjour de 18 heures à 37°C., il existe un précipité important dans les tubes contenant les Tweens 20, 40 et 60 et un précipité moins abondant dans le tube contenant le Tween 80. La solution à l'Emullat R40 ne précipite légèrement qu'après 48 heures. Toutes les solutions témoins restent parfaitement limpides après 5 jours. Il est possible de démontrer au microscope la présence de cristaux dans tous les tubes contenant lipase, détersif et chlorure de calcium.

d) Conclusion.

La lipase de germe de blé est très active sur les Tweens 20, 40 et 60, soit les dérivés des acides laurique, stéarique et palmitique, un peu moins sur le Tween 80 dérivé de l'acide oléique et peu active sur l'Emullat R40, dérivé de l'acide ricinoléique. L'Emullat 06 a été écarté de cette expérience car il donne une solution trouble en bouillon. Les mêmes résultats ont pu être obtenus lorsque la solution de lipase à 1 % est déposée sur du milieu de SIERRA contenu en boîte de Pétri stérile suivant le procédé décrit ci-dessous pour la mise en évidence des lipases d'origine microbienne.

3. ETUDE EXPERIMENTALE.

a. **Réactifs.** Tweens 20, 40, 60 et 80 (Atlas Powder Comp., fournis par la Société Générale des Produits Chimiques, Bruxelles) en solution aqueuse à 10%; Emullat R40 et Emullat 06 (Union Chimique Belge, Ruisbroeck) à la même dilution. Stérilisation 120°C. / 20 minutes.

b. **Milieu.** Il s'agit du milieu de SIERRA non modifié, soit: Difco-Bacto peptone 10,0 g, NaCl 5,0 g, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 0,1 g, eau distillée 1000 cc. Gélosé à 2% et ajusté à $\text{pH} = 7,4$.

Pour chaque souche microbienne, une série de six boîtes de Pétri stériles est préparée. Dans chaque boîte, 1,5 cc de la solution du détersif envisagé est versé stérilement. Ensuite, 15 cc du milieu de base sont répartis et le mélange est soigneusement homogénéisé. Lorsque le milieu est refroidi et solidifié, 2 à 3 gouttes d'une suspension en eau physiologique d'un bouillon de 24 heures de la culture de la bactérie en expérience sont déposées sur la surface de la gélose

et étalées grossièrement à l'aide d'une baguette de verre stérile afin d'obtenir des colonies isolées.

Les boîtes sont ensuite mises à l'étuve à 37°C. et observées quotidiennement. Lors d'essais préliminaires, il a été confirmé:

1. que les boîtes de Pétri non ensemencées et laissées à l'étuve à 37°C. pendant trois semaines ne présentent aucune altération susceptible d'en imposer pour une précipitation du milieu;

2. que l'ensemencement par des souches à activité lipolytique des boîtes de Pétri contenant du détersif en l'absence de calcium ou du calcium sans détersif ne donne pas lieu à précipitation.

c) Etude de l'activité lipolytique du *M. pyogenes* var. *aureus*.

1. Origine des souches: les souches de *M. pyogenes* var. *aureus* proviennent de sources variées afin d'essayer d'établir s'il existe un rapport entre leur activité lipolytique et leur origine. C'est ainsi que 50 souches sont issues des cas les plus variés d'infection et 6 souches, de contamination d'aliments conservés (voir tableau 2).

2. Activité lipolytique suivant le détersif (56 souches, voir tableau 1): les milieux aux Tweens 20, 40 et 60, sont les plus souvent précipités et se comportent à peu près de la même manière vis-à-vis des souches de staphylocoque doré étudiées. Le Tween 80 et l'Emullat 06 sont moins souvent et généralement attaqués plus lentement par les enzymes microbiennes. Le staphylocoque doré est plus indifférent vis-à-vis de l'Emullat R40, bien qu'il convienne de souligner l'apparition d'une réaction positive dans 70% des cas environ.

Si l'on s'attache maintenant au temps nécessaire à la manifestation du pouvoir lipolytique, il faut remarquer que 60% environ des souches sont actives en 24 heures pour les Tweens et 50% environ pour les Emullats.

Dans quelques cas, l'apparition de halos autour de toutes les colonies d'une souche est précédée, un à trois jours auparavant, de la présence de halos autour de quelques colonies seulement. C'est ainsi que pour la souche 3-W, isolée d'un cas de parotidite, il existait, le 6ième jour après l'ensemencement, un halo autour de quatre colonies du milieu au Tween 80, alors que toutes les colonies donnaient une réaction positive au neuvième jour.

3. **Activité lipolytique suivant la souche** de *M. pyogenes* var. *aureus* (56 souches, voir tableau 2).

Les souches de staphylocoque doré peuvent être, suivant leur activité lipolytique, réparties en sept groupes.

Dans le premier groupe (groupe 1), le plus important, se trouvent les souches dont les colonies présentent, dans les 24 heures, des halos opaques pour tous les détersifs de la série (28 souches).

Dans le deuxième groupe (groupe 2), l'apparition d'une précipitation demande plus de 24 heures pour un ou plusieurs détersifs, mais tous sont finalement précipités (9 souches).

Dans le troisième groupe (groupe 3), présence d'halos opaques pour tous les détersifs, à l'exception de l'Emullat R40. Il faut souligner que, très généralement dans ce groupe ainsi que dans les groupes suivants, la réaction demande plus de 24 heures pour les détersifs attaqués (7 souches).

Dans le quatrième groupe (groupe 4), le Tween 80 et l'Emullat R40 ne donnent pas lieu à réaction (4 souches).

Dans le cinquième groupe (groupe 7), absence d'activité lipolytique vis-à-vis du Tween 80, de l'Emullat 06 et de l'Emullat R40 (3 souches).

Dans le sixième groupe (groupe 8), absence d'halos opaques pour les Tweens 40 et 60 et l'Emullat 06 (1 souche).

Enfin, dans le septième groupe (groupe 11), aucune activité lipolytique n'est décelée par la méthode de SIERRA.

L'examen du tableau 2 permet de mettre en évidence les faits suivants:

1) l'activité lipolytique varie fortement suivant les souches de *M. pyogenes* var. *aureus*: elle va d'une activité maximum avec précipitation de tous les milieux en 24 heures à une inactivité totale vis-à-vis des détersifs utilisés.

2) il semble exister un certain rapport entre la virulence et le pouvoir lipolytique. En effet, la plupart des souches isolées de cas pathologiques se retrouvent parmi les groupes à activité lipolytique marquée, tandis que celles provenant d'aliments conservés ont une activité faible ou nulle. Il faut spécialement noter que les trois souches de staphylocoque doré isolées de cas de septicémie appartiennent au premier groupe, c'est-à-dire à celui où l'activité enzymatique est à son maximum, tandis que dans le groupe à activité nulle (groupe 11) ne se rencontrent que quatre souches provenant d'aliments conservés.

4. Comparaison entre l'activité lipolytique et d'autres caractères du *M. pyogenes* var. *aureus* (56 souches, voir tableau 2).

L'activité lipolytique a été mise en parallèle avec d'autres propriétés du staphylocoque doré, soit :

1) la production d'une coagulase pour le plasma sanguin.

2) le caractère hémolytique beta vis-à-vis d'une gélose au sang humain.

3) la liquéfaction de la gélatine.

4) la fermentation de la mannite.

80% environ des souches possèdent une coagulase plus ou moins active pour le plasma sanguin, ce qui est conforme aux données de la littérature. Bien qu'il n'existe pas de parallélisme parfait entre la présence de coagulase et l'activité lipolytique, il faut cependant noter que les quatre souches sans activité lipasique ne possèdent pas de coagulase.

La production de la coagulase a été recherchée dans des conditions constantes de la manière suivante :

100 mg de plasma desséché Bacto-Coagulase Difco sont dissous dans 3,0 cc d'eau distillée stérile. Ces 3 cc sont répartis dans six tubes à hémolyse stériles à raison de 0,5 cc par tube. On y ajoute deux gouttes d'un bouillon de culture de 16 à 24 heures de la souche considérée. La présence d'une coagulation une heure après le début de la mise en contact est indiquée par + + +, deux heures après par + + et trois heures après par +. L'absence de coagulase est marquée par 0.

L'absence du pouvoir hémolytique a été constatée dans 4 cas sur 56 ; l'absence de fermentation de la mannite, également dans 4 cas sur 56 et l'absence de liquéfaction de la gélatine dans 3 cas sur 56. Les quatre souches sans activité lipasique et formant le groupe 11 étaient particulièrement atypiques : ainsi, la souche I-J ne fermentait pas la mannite, la souche 2-I n'était pas hémolytique et ne fermentait pas la mannite, la souche 4-K, anhémolytique ne liquéfiait pas la gélatine et la souche 2-I n'était pas hémolytique. Ces quatre souches étaient isolées d'échantillons de viande conservée et ne possédaient pas de coagulase.

Il semble exister un certain parallélisme entre la présence d'une coagulase et l'activité lipolytique. En effet, l'absence de coagulase se rencontre surtout pour des souches à pouvoir lipolytique faible ou nul.

d) **Activité lipolytique d'autres espèces microbiennes** (voir tableau 2; 19 souches).

Pour classer les autres espèces microbiennes suivant leur activité lipolytique, il a été nécessaire de créer 4 nouveaux groupes, à savoir: un groupe 5 pour deux souches de *M. pyogenes* var. *albus* ne précipitant pas les milieux à l'Emullat 06 et à l'Emullat R40, un groupe 6 pour une souche de *Pseudomonas aeruginosa* n'attaquant pas les Tweens 20 et 80, un groupe 9 pour une autre souche de *Pseudomonas aeruginosa* indifférente vis-à-vis des Tweens 20, 80 et de l'Emullat R40 et, enfin, un groupe 10 pour une souche de *Sarcina flava* active vis-à-vis du Tween 20 et de l'Emullat R40 seulement.

Six souches de *M. pyogenes* var. *albus*, de diverses origines ont été isolées. L'activité lipasique présente la même diversité qu'avec les souches de staphylocoque doré: une souche isolée d'un cas de folliculite relève du groupe 1, c'est-à-dire que tous les détersifs sont attaqués en 24 heures; deux autres souches, provenant respectivement d'un cas de septicémie et d'une dermite pubienne attaquent tous les détersifs, sauf les Emullats (groupe 5); enfin, trois souches n'ont aucune activité lipolytique et proviennent, la première d'un cas d'acné, la deuxième d'une ulcération cutanée et la dernière d'une contamination alimentaire (groupe 11). Il faut noter à nouveau, que la souche isolée d'aliment n'a pas d'activité lipasique. Le staphylocoque d'Oxford précipite en 24 heures les milieux aux Tweens 20, 40, 60 et à l'Emullat 06, en 48 heures, le Tween 80 et en cinq jours, l'Emullat R40 (groupe 2).

Cinq souches de *M. epidermidis* se répartissent en trois groupes. L'activité lipolytique est donc toujours aussi variée. A titre de contrôle, la recherche de coagulase pour le plasma sanguin a été négative pour ces cinq souches.

Les autres espèces isolées comprennent deux souches de *Pseudomonas aeruginosa*, une d'*E. coli*, une de *S. flava*, une de *G. tetragena*, une de *M. aurantiacus* et une de *M. flavus*.

La note dominante de l'activité lipasique paraît, par conséquent, être la diversité.

IV. CONCLUSION.

La détermination de l'activité lipolytique des bactéries par la méthode de SIERRA (1957) permet, semble-t-il, de mettre en évidence une certaine spécificité dans l'activité lipasique. Peut-être pourrait-on parler de laurase, palmitase, stéarase, oléase et ricinoléase.

TABLEAU 1.

Délai d'apparition d'halos opaques autour des colonies microbiennes
(56 souches de *M. pyogenes* var. *aureus*).

détergents	précipitation			absence de précipitation	total des souches
	en un jour	en 2 à 5 jours	en plus de 5 jours		
Tween 20	35	13	4	4	56
Tween 40	35	13	3	5	56
Tween 60	36	10	5	5	56
Tween 80	33	8	4	11	56
Emullat 06	30	15	3	8	56
Emullat R40	29	6	3	17	56

TABLEAU 2.

Classification, suivant l'activité lipasique, de 56 souches de *M. pyogenes* var. *aureus*, de 6 souches de *M. pyogenes* var. *albus* et de 13 souches d'espèces microbiennes diverses.

Groupe 1. Présence d'halos opaques dans les 24 heures, pour tous les détergents (28 souches de *M. pyogenes* var. *aureus* et 1 souche de *M. pyogenes* var. *albus*).

Souche	Origine	T20	T40	T60	T80	E06	R40	Coagu- lase	Hé- molyse	Gélatine	Mannite
2-H	Septicémie	++(1)	++(1)	+(1)	++(1)	+(1)	+(1)	+++	+	+(4)	+(1)
2-G	Septicémie	+(1)	++(1)	+(1)	++(1)	+(1)	+(1)	+++	+	+(5)	+(1)
2-K	Septicémie	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+++	+	+(1)	+(1)
1-M	Angine	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+++	+	+(2)	+(2)
1-O	Angine	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+++	+	+(3)	+(2)
3-C	Angine	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	++	+	+(2)	+(1)
3-D	Angine	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	++	+	+(1)	+(1)
4-A	Angine	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	++	+	+(3)	+(1)
1-I	Anthrax	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+++	+	+(2)	+(2)
2-U	Furoncle	++(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+	+	+(1)	+(2)
3-O	Furoncle	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	0	+	+(5)	+(1)
3-R	Furoncle récidivant lèvre supérieure	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	0	+(2)	+(11)	+(1)
4-M	Furonculose récidivante	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+++	+	+(1)	+(6)
5-D	Furoncle du bras	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	++	+	+(1)	+(1)
5-F	Furoncle de la lèvre supérieure	++(1)	++(1)	+(1)	++(1)	+(1)	+(1)	+	+(2)	+(1)	+(2)
5-L	Furoncle	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+	+	+(1)	+(1)
5-O	Furoncle	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	—	+	+(1)	+(1)
5-P	Pyodermite	++(1)	++(1)	+(1)	+(1)	+(1)	+(1)	—	+	+(1)	+(3)
4-P	Abcès	++(1)	++(1)	+(1)	++(1)	+(1)	+(1)	+++	+	+(1)	+(2)
2-Y	Impétigo	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+	+	+(1)	+(1)
5-K	Impétigo	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	++	+(2)	+(1)	+(1)
5-H	Sycosis de la barbe	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	++	0	+(1)	+(1)
4-B	Périonyxis purulent	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+++	+	+(9)	+(1)
4-H	Pustules plantaires	+(1)	++(1)	+(1)	+(1)	+(1)	+(1)	+++	+	+(8H)	+(1)
4-L	Ulcère de jambe	++(1)	++(1)	+(1)	+(1)	+(1)	+(1)	0	+	+(1)	+(1)
4-G	Eczéma impétigé nise des doigts	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+	+	+(1)	+(1)
2-C	Gangrène diabétique	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+++	+	+(3)	+(1)
3-E	Diabète apais antibiotiques	++(1)	+(1)	+(1)	+(1)	+(1)	+(1)	++	+	+(2)	+(1)
5-E	<i>M. pyogenes</i> var. <i>albus</i> (Folliculite)	++(1)	++(1)	+(1)	++(1)	+(1)	+(1)	++	+(2)	+(2)	+(1)

Groupe 2. Présence d'halos opaques pour tous les détersifs, mais en plus de 24 heures pour un ou plusieurs détersifs (13 souches, dont 9 de *M. pyogenes* var. *aureus*).

Souche	Origine	T20	T40	T60	T80	E06	R40	Coagu- lase	Hé- molyse	Gélatine	Mannite
4-N	Angine	+(1)	+(1)	+(1)	+(1)	+(1)	+(2)	+++	+	+(1)	+(2)
1-E	Abcès	++(1)	+(1)	+(1)	+(1)	+(2)	+(1)	+++	+	+(2)	+(1)
1-F	Furoncle	++(1)	+(1)	+(1)	+(1)	+(2)	+(2)	+++	+	+(7)	+(2)
3-M	Pustules du pied	+(1)	+(1)	+(1)	+(1)	+(2)	+(2)	—	+	+(8)	+(1)
3-P	Furoncle	+(1)	+(1)	+(1)	+(3)	+(3)	+(5)	++	+	+(5)	+(1)
1-G	Pyodermite	+(1)	+(1)	+(2)	+(2)	+(2)	+(4)	++	+	+(2)	+(1)
*1-L	Conjonctivite aiguë	+(3)	+(2)	+(2)	+(3)	+(2)	+(3)	+++	+	0	0
3-W	Parotidite	+(2)	+(2)	+(1)	+(9)	+(8)	+(9)	++	+	+(13)	+(1)
1-N	Impétigo	+(2)	+(2)	+(2)	+(6)	+(3)	+(15)	+++	+	+(1)	+(2)
Stock	Staphylocoque d'Oxford	+(1)	+(1)	+(1)	+(2)	+(1)	+(5)				
4-F	<i>M. epidermidis</i> (eczématides)	+(1)	+(1)	+(1)	+(2)	+(9)	+(9)				
4-V	<i>M. epidermidis</i> (ectyma)	+(1)	+(1)	+(1)	+(9)	+(9)	+(8)				
1-X	<i>Escherichia coli</i> (gangrène artéritique)	+(2)	+(2)	+(2)	+(9)	+(2)	+(10)				

Groupe 3. Présence d'halos opaques pour tous les détersifs, sauf l'Emullat R 40 (7 souches de *M. pyogenes* var. *aureus*).

Souche	Origine	T20	T40	T60	T80	E06	R40	Coagu- lase	Hé- molyse	Gélatine	Mannite
3-S	Furoncle du cuir chevelu	+(1)	+(1)	+(1)	+(1)	+(1)	0	++	+	+(5)	+(1)
3-F	Furoncle du menton	+(2)	+(2)	+(2)	+(3)	+(3)	0	—	+	+(5)	+(1)
4-O	Angine	+(2)	+(3)	+(2)	+(4)	+(4)	0	+	+	+(1)	+(2)
3-A	Pyodermite et eczéma	+(3)	+(3)	+(1)	+(6)	+(6)	0	++	+	+(1)	+(2)
2-F	Abcès	+(2)	+(2)	+(3)	+(8)	+(2)	0	—	+	+(3)	+(1)
2-M	Plaie infectée	+(5)	+(3)	+(11)	+(5)	+(3)	0	+++	+	+(2)	+(1)
2-W	Pus rénal	+(2)	+(5)	+(3)	+(10)	+(7)	0	++	+	+(3)	+(1)

Groupe 4. Présence d'halos opaques pour tous les détersifs, sauf le Tween 80 et l'Emullat R40 (4 souches de *M. pyogenes* var. *aureus* et 1 souche de *M. epidermidis*).

Souche	Origine	T20	T40	T60	T80	E06	R40	Coagu- lase	Hé- molyse	Gélatine	Mannite
1-H	Impétigo	+(2)	+(4)	+(3)	0	+(3)	0	+	+	+(1)	+(2)
4-X	Intertrigo axillaire	+(3)	+(3)	+(3)	0	+(5)	0	+++	+	+(1)	+(1)
4-D	Surinfection d'une réticulendothéliose	+(3)	+(5)	+(5)	0	+(3)	0	0	+	+(1)	+(2)
2-E	Diarrhée après antibiotique	+(4)	+(22)	+(13)	0	+(4)	0	0	+	0	+(1)
3-Y	<i>M. epidermidis</i> (Kyste sébacé infecté)	+(3)	+(3)	+(3)	0	+(5)	0				

Groupe 5. Présence d'halos opaques pour tous les détersifs, sauf l'Emullat 06 et l'Emullat R40 (2 souches de *M. pyogenes* var. *albus*).

Souche	Origine	T20	T40	T60	T80	E06	R40	Coagu- lase	Hé- molyse	Gélatine	Mannite
3-J	<i>M. pyogenes</i> , var. <i>albus</i> (Septicémie)	+(2)	+(2)	+(1)	+(10)	0	0	+++	+	+(3)	+(1)
*4-Z	<i>M. pyogenes</i> , var. <i>albus</i> (Dermite pubienne)	+(2)	+(2)	+(3)	+(7)	0	0	++	0	0	+(1)

Groupe 6. Présence d'halos opaques pour tous les détersifs, sauf les Tweens 20 et 80.

Souche	Origine	T20	T40	T60	T80	E06	R40
2-A	<i>Pseudomonas aeruginosa</i> (plaie de mastéctomie)	0	+(4)	+(4)	0	+(5)	+(4)

Groupe 7. Absence d'halos opaques pour le Tween 80, l'Emullat 06 et l'Emullat R40 (3 souches de *M. pyogenes* var. *aureus*).

Souche	Origine	T20	T40	T60	T80	E06	R40	Coagu- lase	Hé- molyse	Gélatine	Mannite
1-W	Pus de péritonite	+(9)	+(8)	+(8)	0	0	0	+++	+	+(3)	+(2)
2-D	Gangrène diabétique	+(14)	+(8)	+(8)	0	0	0	++	+	+(3)	+(1)
*2-O	Lait en poudre	+(6)	+(3)	+(6)	0	0	0	0	+	+(2)	0

Groupe 8. Absence d'halos opaques pour les Tweens 40 et 60 et l'Emullat 06 (1 souche de *M. pyogenes* var. *aureus*).

Souche	Origine	T20	T40	T60	T80	E06	R40	Coagu- lase	Hé- molyse	Gélatine	Mannite
1-P	Echantillon de viande	+(13)	0	0	+(5)	0	+(12)	+++	+	+(2)	+(1)

Groupe 9. Absence d'halos opaques pour les Tweens 20 et 80 et l'Emullat R40.

Souche	Origine	T20	T40	T60	T80	E06	R40
4-S	<i>Pseudomonas aeruginosa</i> (Intertrigo axillaire)	0	+(3)	+(3)	0	+(3)	0

Groupe 10. Absence d'halos opaques pour les Tweens 40, 60 et 80 et l'Emullat 06.

Souche	Origine	T20	T40	T60	T80	E06	R40
3-U	<i>Sarcina flava</i> (échantillon de viande)	+(4)	0	0	0	0	+(4)

Groupe 11. Absence d'halos opaques pour tous les détersifs (12 souches dont 4 *M. pyogenes* var. *aureus* et 3 *M. pyogenes* var. *albus*).

Souche	Origine	Coagulase	Hémolyse	Gélatine	Mannite
*1-J	Echantillon de viande	0	+	+(4)	0
*2-I	Echantillon de viande	0	0	+(1)	0
2-J	Echantillon de viande	0	0	+(1)	+(2)
*4-K	Echantillon de viande	0	0	0	+(2)
3-Z	<i>M. pyogenes</i> var. <i>albus</i> (acné)	0	0	0	+(1)
4-Y	<i>M. pyogenes</i> var. <i>albus</i> (ulcération)	0	0	0	+(1)
4-C	<i>M. pyogenes</i> var. <i>albus</i> (viande)	0	0	+(2)	+(1)
2-P	<i>M. epidermidis</i> (balanoposthite)				
3-I	<i>M. epidermidis</i> (réticuloendothéliose)				
3-N	<i>G. tetragena</i> (dermite de l'oreille)				
3-G	<i>M. aurantiacus</i> (viande)				
3-H	<i>M. flavus</i> (viande)				

Nombre de souches de *M. pyogenes* var. *aureus* appartenant à chaque groupe.

Détergents précipités	T ₂₀ , T ₄₀ , T ₆₀ , T ₈₀ , E ₀₆ , R ₄₀		T ₂₀ , T ₄₀ , T ₆₀ , T ₈₀ , E ₀₆	T ₂₀ , T ₄₀ , T ₆₀ , E ₀₆	T ₂₀ , T ₄₀ , T ₆₀	T ₂₀ , T ₈₀ , R ₄₀	Aucun
Groupe	Groupe 1	Groupe 2	Groupe 3	Groupe 4	Groupe 7	Groupe 8	Groupe 9
Nombre de souches "normales"	27	8	7	3	2	1	0
Nombre de souches "atypiques"	1	1	0	1	1	0	4
Total	28	9	7	4	3	1	4

T₂₀ = Tween 20; T₄₀ = Tween 40; T₆₀ = Tween 60; T₈₀ = Tween 80; E₀₆ = Emullat 06; R₄₀ = Emullat R40

*: indique une souche atypique.

Halos	++: précipitation avec cristaux visibles à l'oeil nu. +: précipitation avec cristaux visibles au microscope. 0: absence de précipitation après 30 jours. (...): nombre de jours nécessaires à l'obtention de l'effet.
Coagulase	+++ : coagulation du plasma en une heure. ++ : coagulation du plasma en deux heures. + : coagulation du plasma en trois heures. 0: absence de coagulation du plasma.
Hémolyse	+: hémolyse du type beta, visible en 24 heures sur gélose au sang humain.
Gélatine	+: liquéfaction de la gélatine. 0: absence de liquéfaction de la gélatine en trente jours. (...): nombre de jours nécessaires pour obtenir une liquéfaction.
Mannite	+: fermentation de la mannite à 1%. Indicateur pourpre de bromocrésol. (...): nombre de jours nécessaires pour obtenir la fermentation. 0: absence de fermentation après cinq jours.

Appliquée au *M. pyogenes* var. *aureus*, cette méthode souligne la disparité des souches et paraît indiquer un rapport entre l'activité lipolytique et la virulence. Le contraste entre l'activité très intense des souches isolées de septicémie et celle, nulle ou faible, des souches provenant d'aliments conservés est particulièrement frappant.

Cette nouvelle technique paraît pouvoir être employée, comme complément, à côté de la recherche de la coagulase, dans la détermination de la virulence du *M. pyogenes* var. *aureus*.

R é s u m é.

La méthode de SIERRA, permettant de déterminer l'activité lipolytique des bactéries, a été appliquée à 75 souches microbiennes, dont 56 *M. pyogenes* var. *aureus*. Outre les détergents utilisés par SIERRA, soit les Tweens 20, 40, 60 et 80, deux autres détergents, l'Emullat 06 et l'Emullat R40 ont été employés. L'activité de la lipase de germe de blé a également été étudiée vis-à-vis des détergents. Il est possible de classer les souches de *M. pyogenes* var. *aureus*, de *M. pyogenes* var. *albus* et de *M. epidermidis* en plusieurs groupes en fonction de l'activité lipasique. Cette dernière paraît en rapport avec la virulence microbienne décelée par l'origine des souches et la présence de coagulase.


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